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SOMMAIRE – INHALT – SOMMARIO – CONTENTS

H. MOSER: Modern Approaches to the Study of Mammalian Cells in Culture 385

E. A. ZELLER: Studies on the Active Center of Monoamine Oxidase 399

Brèves communications - Kurze Mitteilungen - Brevi comunicazioni - Brief Reports

J. M. WILSON: Electron Impact and Molecular Dissociation Part IV 403

D. W. MATHIESON, B. JAQUES, G. T. CHAPMAN, V. P. ARVA, and B. G. ENGEL: The Structure of Cassamine and Erythrophlamine 404

J. JELJASZEWICZ and K. WŁODARCZAK: Inhibitory Action of some Compounds on Staphylococcal Coagulase 406

M. GUBAŃSKI and R. KURSTAK: Glutamic Acid-Alanine Transaminase in Tobacco Leaves Infected with TMV. 407

A. VESTERMARK and H. BOSTRÖM: 10. On the Sulphurylation of Mono-, Di-, and Trihydric Phenols 408

H. K. MITCHELL, P. S. CHEN, and E. HADORN: Tyrosine Phosphate on Paper Chromatograms of *Drosophila melanogaster*. 410

K.-B. AUGUSTINSSON: The Esterase Activity of Dog's Colostrum 411

P. SENSI, M. T. TIMBAL, and G. MAFFII: Rifomycin IX. Two New Antibiotics of Rifomycin Family: Rifomycin S and Rifomycin SV. Preliminary Report 412

U. PARENTI: Self-Fertilization in *Ophryotrocha labronica* . . . 413

A. HOFMANN und A. TSCHERTER: Isolierung von Lysergsäure-Alkaloiden aus der mexikanischen Zauberdroge Ololiuqui (*Rivea corymbosa* (L.) Hall. f.) 414

M. PRIVAT DE GARHILHE, C. GROS, J. PORATH, and EVA-BRITA LINDNER: Further Studies on Corticotropin Releasing Factor (CRF); Corticotropin Releasing Activity of Synthetic Peptides 414

H. KAPPELER and R. SCHWYZER: Synthetic Peptides Related to the Corticotropins (ACTH) and the Melanophore Stimulating Hormones (MSH) Possessing Corticotropin Releasing Activity (CRF-Activity) 415

B. C. R. STRÖMBLAD: Adrenaline-Noradrenaline Content of the Submaxillary Gland of the Cat 417

Å. BERTLER, A.-M. ROSENGREN, and E. ROSENGREN: In Vivo Uptake of Dopamine and 5-Hydroxytryptamine by Adrenal Medullary Granules 418

Å. BERTLER, N.-Å. HILLARP, and E. ROSENGREN: Storage of New-Formed Catecholamines in the Adrenal Medulla. . . . 419

B. D. JANKOVIĆ and K. ISAKOVIĆ: Immunological Tolerance in Chickens Induced by Blood Group Substance. 420

K. G. PAUL and N. WIQVIST: The Influence of Estradiol, Progesterone, and Relaxin on the Peroxidase Activity in the Uterus 421

G. MAFFII, L. FONTANELLA, P. SCHIATTI und E. TESTA: Herstellung und biologische Wirkung des 16α-Methyl-desoxycorticosteronacetats 422

M. CAMPANI, R. CORTINOVIS, and A. ZONTA: Studies on Wound Healing: 1. Metabolism of ^{35}S in 'Repair Tissue' of Skin Wounds during the various Phases of Healing 423

C. G. BUTLER: Queen Recognition by Worker Honeybees (*Apis mellifera* L.) 424

Z. QUTOB: Pressure Perception in *Ostariophysi* 426

R. KATO: Reduced Sensitivity to some Drugs 48 h after Chlorpromazine Treatment 427

J. LOWENTHAL, J. A. MACFARLANE, and K. M. MCDONALD: The Inhibition of the Antidotal Activity of Vitamin K, against Coumarin Anticoagulant Drugs by its Chloro Analogue. . 428

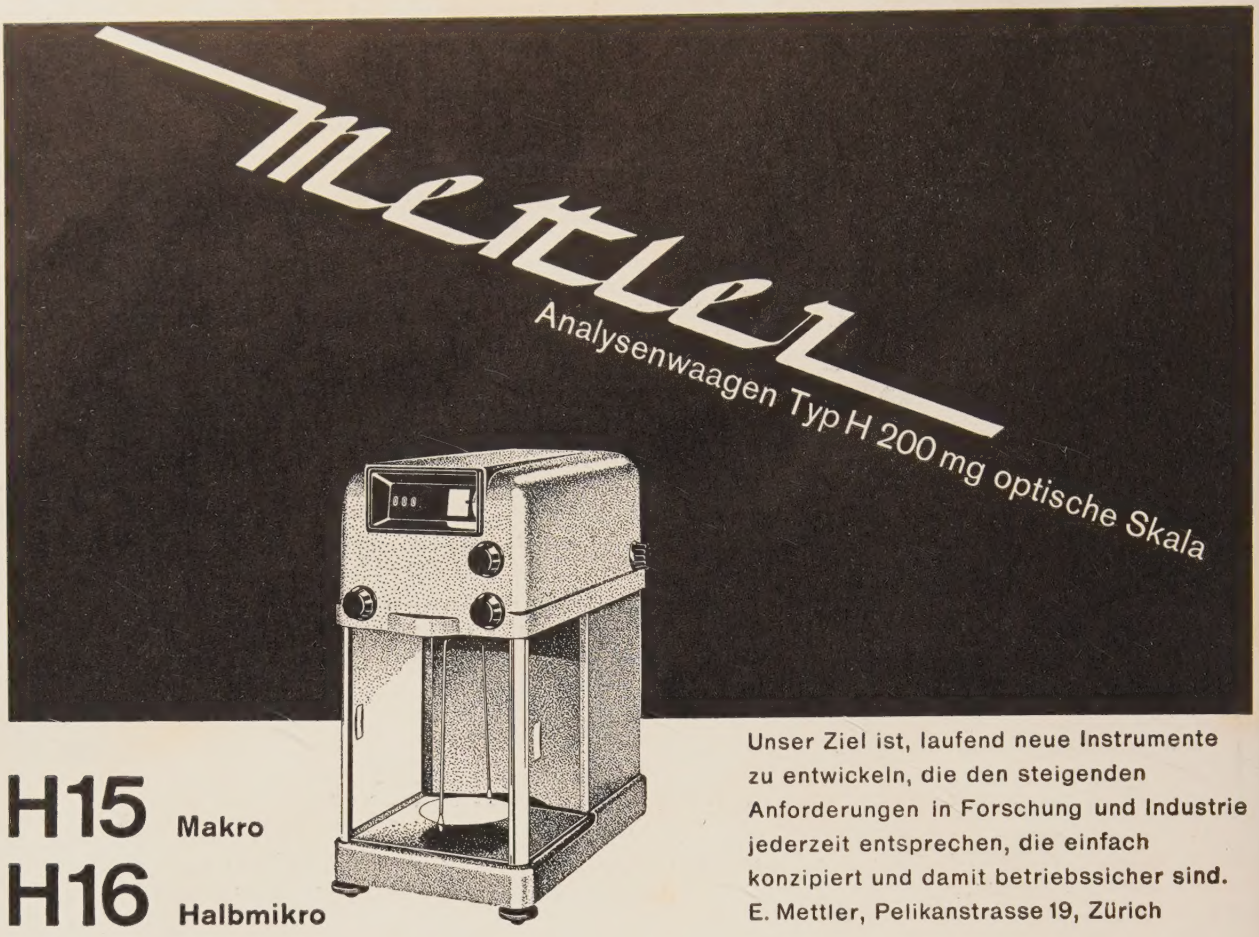
C. CAVALLERO, G. CHIAPINO, F. MILANI, and E. CASELLA: Uptake of ^{35}S Labelled Sulfate in the Exorbital Lacrymal Glands of Adult and Newborn Rats under Different Hormonal Treatment 429

MARIA BASSI and A. BERNELLI-ZAZZERA: Effects of Potassium Ions on Brain Respiration and Aminoacid Incorporation into Brain Proteins *in vitro* 430

H. RINDERKNECHT: A New Technique for the Fluorescent Labelling of Proteins (Pro Experimentis) 430

H. V. LOTT: Eine einfache Apparatur zur serienweisen Redoxtitration (Pro Laboratorio) 431

A. WETTSTEIN: Fortschritte der Arzneimittelforschung (Acta Comparata) 432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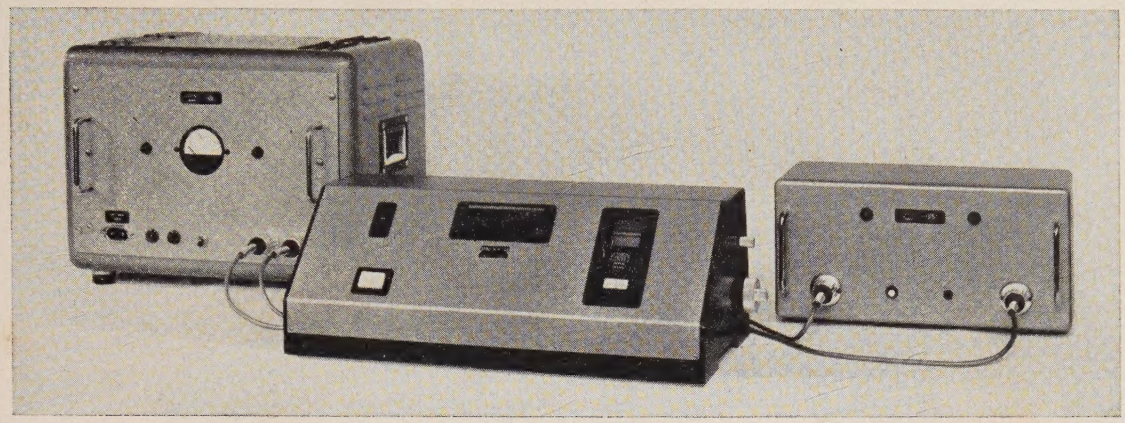

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Modern Approaches to the Study of Mammalian Cells in Culture*

By H. MOSER**

The development of powerful new culture techniques has opened up novel approaches to the study of mammalian cells *in vitro*. These modern techniques permit many of the quantitative experimental approaches which are possible with many micro-organisms to be applied now to biochemical and genetical studies of single mammalian cells *in vitro*. It is the purpose of this short article to introduce the reader to some aspects of modern cell culture technology and to summarize some of the studies on mammalian cells carried out by the author and other research workers.

Modern Cell Culture Techniques

1. Chemical Dissociation of Mammalian Cells. The liberation of large numbers of single mammalian cells from tissue fragments or tissue cultures by chemical means without appreciable loss of viability has been a significant achievement[1–5]. This technique involves the brief incubation of solid tissue or of tissue cultures in balanced salt solutions deficient in Ca^{++}, Mg^{++}, and $H_2PO_4^-$ at 37°C, in the presence of trypsin, mixtures of tryptic enzymes (pancreatine) or in the presence of a chelating agent (Versene). Final dissociation is achieved mechanically and may yield cell suspensions composed of up to 90% single cells.

2. Partially defined and defined growth media for mammalian cells. The conventional tissue and cell culture media are prepared by combining electrolyte solutions ('balanced' salt solutions) with complex and uncharacterized ingredients such as lymph, plasma, and tissue extracts (notably chick embryo extract) or ultrafiltrates of the latter. These complex, undefined nutrients, do not permit exact biochemical and physiological studies. Moreover, since they introduce into the nutrient 'cytotoxic' compounds along with essential growth factors, they are suboptimal growth media for mammalian cells. Thus the formulation of *chemically defined background media* composed of electrolytes, carbohydrates, amino acids, vitamins, and other purified compounds (ROSENBERG's S 27[6], FISHER's V 614[7], EAGLE's Basal Mixture[8,9], WHITE's medium[10,11], MORGAN's 199 and M 150[12,13], HEALEY's 703[14], MORGAN's M 416[15], HEALEY's 858[16], EVANS' NCTC-107[17] and -109) made possible many new approaches to physiological, biochemical, and genetic studies of mammalian cells *in vitro*.

The *growth media* (complete media) of today can be classified as four groups[18]:

a) Serum supplemented growth media, i.e. chemically defined background media supplemented with 10% to 20% whole adult or fetal mammalian serum. These media are defined except for the molecular (dialyzable) and the macromolecular (non-dialyzable) portion of the serum supplements.

b) Serum protein supplemented growth media, i.e. chemically defined background media supplemented with 5%–10% dialyzed adult or fetal mammalian serum. These growth media are defined except for the

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** Biological Laboratory, Cold Spring Harbor, N. Y. and Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York.

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macromolecular portion of serum (serum protein complex). In these nutrient media the omission of a single essential growth factor produces a nutritional deficiency and early death of the culture.

c) Chemically defined, protein containing, growth media, i.e. chemically defined background media supplemented with certain purified proteins isolated from mammalian sera (protein fractions with ultracentrifugal and electrophoretical homogeneity of at least 97%[19]).

d) Protein-free chemically defined growth media, for example growth medium NCTC-107 of Evans *et al.* for L-929 mouse cells in monolayer culture.

The trend in this area of technology is to provide minimized optimal media, that is media which omit unessential and cytotoxic molecular or macromolecular species and permit the sustained growth of single (widely separated) cells *in vitro*.

3. The control of hydrogen-ion concentration in cell cultures. The rate of mammalian-cell reproduction is a function of the pH of the culture fluid. Maximum reproduction rate, as a function of hydrogen-ion concentration, is attained at pH = 7 or at a pH very close to this value[20]. At pH = 7.6 non-malignant (normal) mammalian cells stop dividing but remain viable (maintenance state)[20]. Since the sustained growth of mammalian cells *in vitro* is very susceptible to small changes in pH, modern cell culture employs refined methods for maintaining the pH within the desired limits or at a fixed value.

Since bicarbonate is the major buffer system of the growth media for mammalian cell cultures, and since actively growing somatic cells produce lactic acid, the pH, at a given temperature and a given atmospheric pressure, is a function of the bicarbonate concentration in the medium, of the partial pressure of CO_2 in the surrounding gas phase, and of the glycolytic activity of the growing cells. At low population densities in stationary cultures or even also at high population densities in perfused agitated cultures the acid production of the organism is negligible. Under such conditions the pH of the culture approximates

$$pH = pK_a + \log ((HCO_3\text{-})/(CO_2))$$

(Henderson-Hasselbach equation) where pK_a is a constant, (CO_2) the molar concentration of CO_2, $(HCO_3\text{-})$ the molar concentration of bicarbonate ion in the culture fluid. Thus since the pK_a of the bicarbonate buffer system is 6.322 at 37°C pH values close to 7 can be maintained by adjusting the partial pressure of CO_2 in the gas phase and the bicarbonate concentration in the culture fluid.

Classic cell culture which employed anaerobic culture devices (tightly stoppered culture vessels) did not permit accurate gas and therefore pH control. Modern cell culture devices are of the aerobic type which are either placed in a humidified atmosphere containing 5% to 10% CO_2 (CO_2 incubator) or which are individually flushed with the gas mixture.

4. Monolayer cell culture. This stationary culture technique[2, 21] omits complex supporting structure (plasma clot, fibrinogen clot) in which cells or tissue explants are embedded. Dissociated cells, in Ca^{++} containing nutrient, at pH = 7 and a temperature close to 37°C adhere directly to the glass surface of the culture vessels (flasks, tubes, Petri-dishes etc.). As they begin to grow they stretch out horizontally on the glass surface and finally form confluent cell sheets consisting of only one layer of cells. In static monolayer culture two distinct morphological cell types are most frequently observed: Fibroblast-like cells, i.e. elongated spindle-shaped cells with needle-like extensions (Fig. 1), and epithelial-like cells, i.e. polygonal-shaped cells (Fig. 2). Prior to mitosis cells of both types assume a round form.

Monolayer-cell cultures prove to be very susceptible to infection by virus. This property has been exploited by Dulbecco and Vogt[3, 22] in the development of their ingenious plaque assay technique for animal viruses.

5. Fluid Suspension Culture Method. The ability to grow dispersed mammalian cells in fluid suspension analogous to the growth of unicellular microorganisms in liquid growth media has been a major achievement. The method involves the inoculation of a dense cell suspension into a growth tube with an agitating mechanism and filled with growth medium deficient or poor in Ca^{++}. Under appropriate conditions of agitation, a pH close to 7, a temperature of 37°C and sustained nutrient supply, the cells begin to multiply suspended mainly as single cells. Throughout the logarithmic growth phase they sustain their rounded form (see Fig. 3). Various types of agitated systems have been suggested as satisfactory for continuous growth of mammalian cells in fluid suspension[22, 23–28].

Undoubtedly the most advanced system is the cytogenerator of Graff and McCarty[20] (see Fig. 4). The cytogenerator is an agitated culture system with perfusion and gas control. The cells produced are retained within a U-shaped growth tube. Fresh nutrient is delivered to the growth tube by perfusion through a sep

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Fig. 1. Fibroblast-like cells derived from adult human skin (normal-diploid strain M-1).

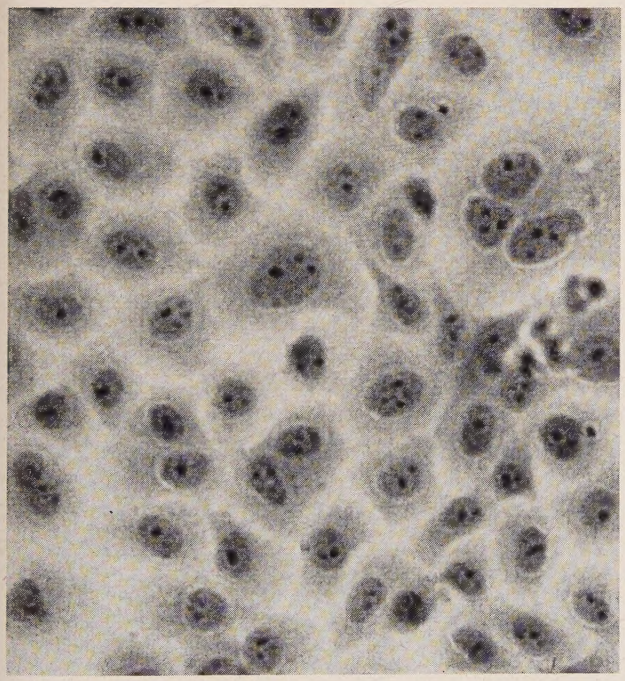

Fig. 2. Epithelial-like cells derived from adult sternal human marrow (altered heteroploid strain Detroit-98C6b).

tum (sintered glass filter), and the spent medium which includes accumulated cell metabolites is removed in a similar fashion. Accurate, matched variable pumps are used to keep outflow rate and inflow rate closely equal in order to maintain the volume of the culture fairly constant. The composition of the gas phase (CO_2-air) is controlled and can be regulated to adjust the pH of the culture – which contains sodium bicarbonate as the buffer – within the desired value. Alternate gas pressure pulses provide sufficient agitation to retain the cells in suspension. Because of its features this system is particularly suited for the quantitative study of cell metabolism and for mass production of mammalian cells and viruses *in vitro*.

Cells of several altered (hyperdiploid) cell strains, notably of the now famous mouse L-fibroblast strain and the Mouse Ascites strain have been successfully grown in the cytogenerator[20]. Figure 5 illustrates the growth of L-929 mouse cells in this system and in a medium consisting of Ca^{++} – free Eagle's Basal Mixture and of 10% adult horse serum. If the pH is kept close to pH = 7, and if the concentration of glucose in the growth tube is maintained above a certain value

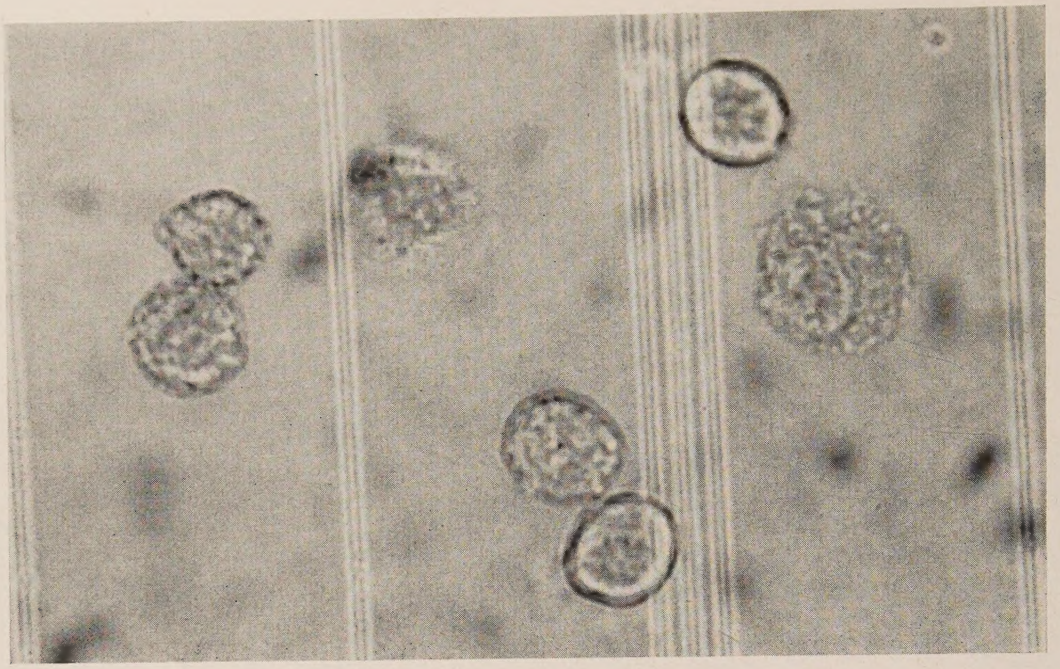

Fig. 3. L929 mouse cells in suspension culture.

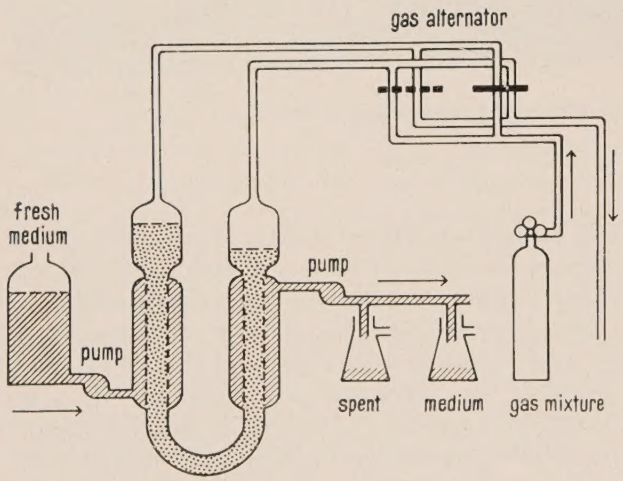

Fig. 4. Schema of the cytogenerator [reproduced with the permission of the author from Trans. N. Y. Acad. Sci. *21*, 505 (1959).

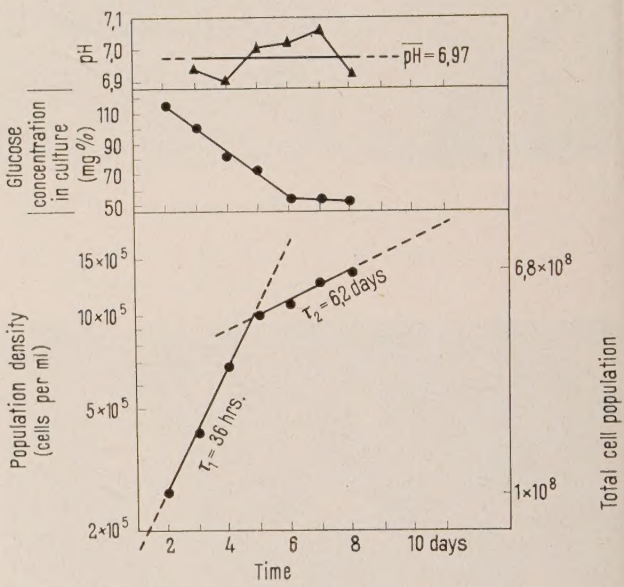

Fig. 5. Growth of L929 mouse cells in the cytogenerator. (Temperature 37°C).

(limiting concentration)[29] the L-929 cells reproduce logarithmically at maximum rate (i.e. at a doubling time approximating 24 h) at 37°C. Attempts are being made now by the author in collaboraton with Dr. S. Graff to propagate unaltered (diploid) human cells in this fashion.

6. The clonal culture method. Plating procedures for single cells derived from man and other mammals analogous to the quantitative plating of bacteria on agar media have been developed by Puck *et al.*[30, 31]. By this method a determined number of carefully trypsinized cells from predominantly mono-disperse cell suspensions are inoculated into Petri-dishes containing liquid growth medium and evenly distributed over the glass surface. When the Petri-dishes are incubated in hori-

[29] This is achieved either by regulating the rate of glucose input (perfusion rate) or regulating the glucose consumption (population density, pH).

[30] T. T. Puck, P. I. Marcus, and S. J. Cieciura, J. exp. Med. *103*, 273 (1956).

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zontal position at 37° C in a humidified atmosphere of CO_2 and air, and the pH adjusted to 7.0 or 7.1, the widely separated single cells attach to the glass and begin to multiply, forming macroscopic two-dimensional colonies upon incubation for 10 to 20 days. Both hyperdiploid (altered) cells of epithelial-like morphology and diploid (unaltered) cells of fibroblast-like morphology can be grown by this method into macroscopic clones. The plating efficiencies obtained with diploid-fibroblast-like cells are generally lower (5% to 40%) than those obtained with epithelial-like hyperdiploid altered epithelial cells[32, 33] (Tab. I), and a more complex growth medium is required for the sustained growth *in vitro* of single cells of the unaltered fibroblast-like variety[34]. The epithelial cells form tightly packed colonies with well-defined edges. In sharp contrast to this the fibroblast-like cells form loosely packed colonies with large surface area and not well-defined contours[35] (see Fig. 6).

Although this method of clonal growth has many limitations and shortcomings, it provides undoubtedly an excellent tool for the screening of large numbers of mammalian cells under controlled conditions for physiological and genetic studies, and a tool for rapid cloning of heterogenous cell cultures.

7. Cloning techniques. The isolation of phenotypic (functional or morphologic) variants such as tumor or hormone producing cells[36] or of genetic variants (mutants) from heterogenous cell cultures is achieved by methods which involve (a) the transfer of an isolated cell to a small volume of growth medium which permits sustained multiplication, or (b) by the isolation of colonies produced by single cells in Petri-dishes or in culture tubes.

Earle *et al.*[37] developed a very exacting technique for the production of clonal cell strains. This technique involves the sealing of individual cells in capillary tubes where diffusible metabolic products essential for multiplication remain in association with the cells. Another technique developed by Vogt and Dulbecco[38] involves the isolation and transfer of an individual cell into a small drop of growth medium deposited under paraffin oil on a Petri-dish. The clones which are formed in the individual-drop cultures can be trypsinized and subcultured for the production of mass cultures.

The method of Puck, Marcus, and Cieciura consists in the direct isolation of well-separated colonies formed by single cells on Petri-dishes[30]. By this method each of the isolated subcultures must be passed through several single-cell isolation procedures in order to insure genetic purity.

A technique of clone isolation developed and tested by the author[18] is based on the distribution of single cells over a large number of tubes inoculated with equal amounts of a monodisperse cell suspension. If 1 ml aliquots of a monodisperse cell suspension containing in the average μ ($\mu \sim 1$) viable cells per unit volume are introduced into a large number of culture tubes with a flat bottom (Leightontype tubes), the fraction of tubes yielding x macroscopic colonies approximates $P(x) = (\mu^x/x!) \exp(-\mu)$ (Poisson distribution, $x = 1, 2, 3 \ldots, \infty$). The fraction, $P(1) = \mu \exp(-\mu) \sim 0.37$, of the tubes contain only a single colony. Thus clones can be isolated by selecting in such an experiment the tubes in which only a single colony appears. In practice, the tubes inoculated with 1 ml aliquots are incubated in horizontal position in the CO_2 incubator, so that there is enough space for the development of several isolated macroscopic colonies. The tube cultures with a single colony are trypsinized and subcultured, and the procedure repeated to insure genetic purity of the isolate.

8. Karyotype analysis in mammalian cell culture. The study of chromosomal constitution of mammalian cells in culture by the chromosome spreading techniques developed by Hsu and Pomerat[39] and others not only permits the convenient determination of the chromosomal constitution of an individual but also provides an exact tool for determining alterations of cells *in vitro* (polyploidy, aneuploidy, chromosome aberrations). The method involves pretreatment of actively growing cultures with colchicine for 'accumu-

Tab. I. Plating efficiencies of heteroploid epithelial-type cell strains in Eagle's Basal Mixture supplemented with 10% adult horse serum.

Human strain	Experiment, number	Incubation time, days	Number of cells plated	Colony counts	Mean colony count	Plating efficiency (in %)
Detroit-98	1	14	219	106 119	113	51.6
Detroit-98C6b	1	15	209	100 135	118	56.6
Detroit-98C6b	2	16	369	277 225 193 185 248	226	61.3
Detroit-98C6b	3	15	242	125 152	139	57.5
D-189	1	16	142	122 87 82	97	68.3
Human Amnion (Hayflick-Fernandez)	1	16	265	120 90 80	97	36.6

[32] H. Moser and K. Tomizawa, Annual Report of the Biological Laboratory, Long Island Biological Association, Cold Spring Harbor, L. I., N. Y. 68th year (1958).

[33] R. Z. Lockhart, Jr. and H. Eagle, Science *129*, 252 (1959).

[34] T. T. Puck, S. J. Cieciura, and A. Robinson, J. exp. Med. *108*, 945 (1958).

[35] T. T. Puck, S. J. Cieciura, and H. W. Fisher, J. exp. Med. *106*, 145 (1957).

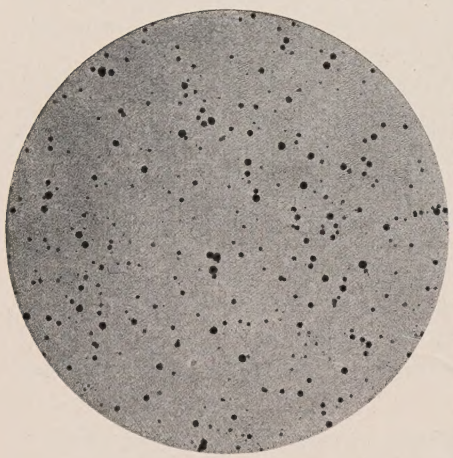
[36] K. W. Thompson, M. M. Vincent, F. C. Jensen, R. T. Price, and E. Schapiro, Proc. Soc. exp. Biol. Med., N. Y. *102*, 403 (1959).

[37] W. R. Earle, Methods med. Res. *4*, 217 (1951).

[38] M. Vogt and R. Dulbecco, Virology *5*, 425 (1958).

[39] T. C. Hsu and C. M. Pomerat, J. Heredity *44*, 23 (1953).

lation' of cells in metaphase, and with hypotonic salt solution for spreading the chromosomes. This method yields preparations where the 46 chromosomes of man can be clearly counted and identified in numerous metaphase plates (see Fig. 10).



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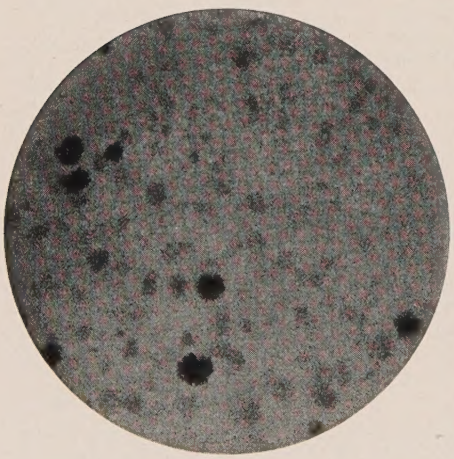

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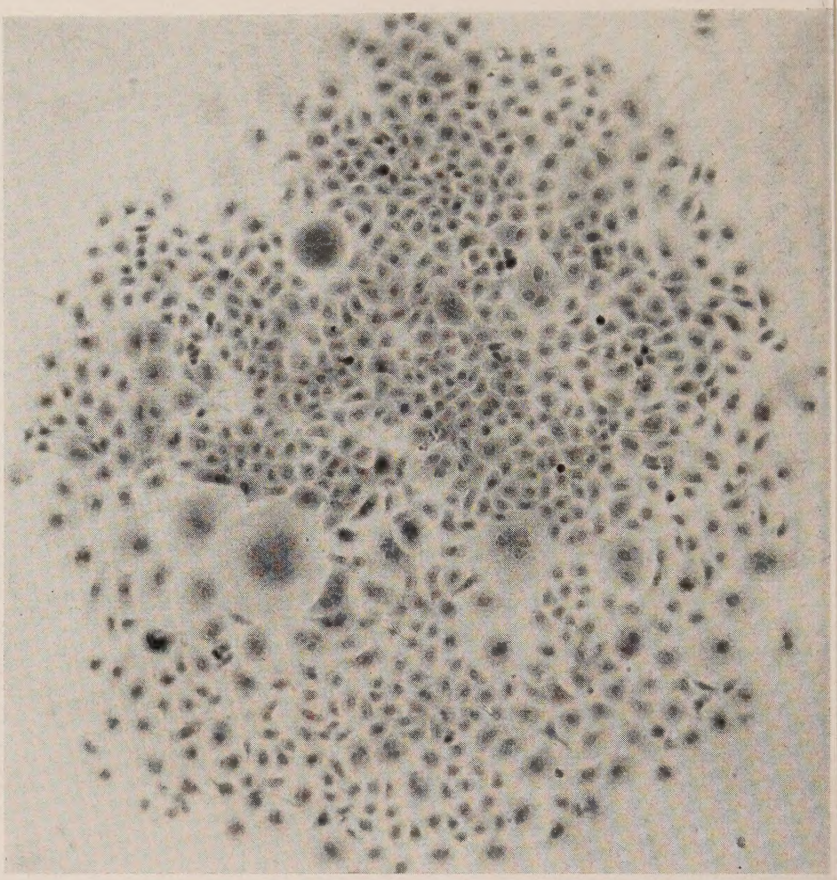

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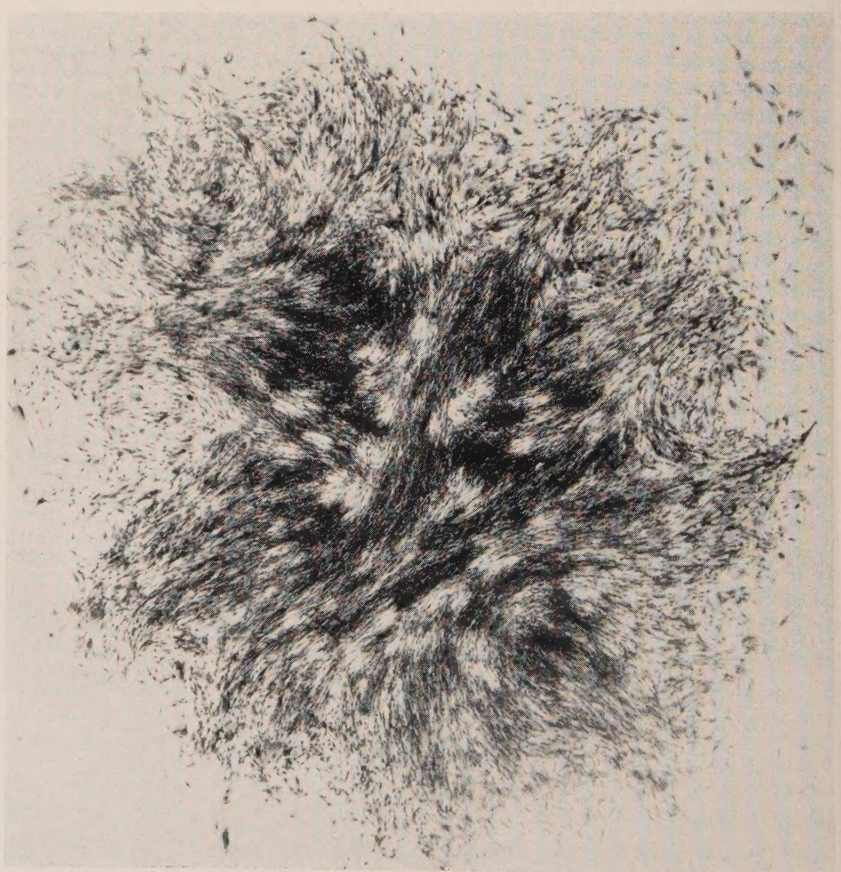

d

Fig. 6. a) Clonal growth of epithelial-like cells (altered-heteroploid strain Detroit-98C6b). b) Clonal growth of fibroblast-like cells (normal-diploid strain M-1). c) A colony formed by an epithelial-like cell (Detroit-98C6b). d) A colony formed by a fibroblast-like cell (M-1).

The Evolution of Mammalian Cell Cultures

1. Adaptation and selection in primary cultures. Practically every tissue of the mammalian organism yields cells which are able to multiply *in vitro* under appropriate conditions of culture. In the past the initiation of cell cultures from biopsy materials has often failed as a result of suboptimal growth media and adverse culture conditions. The introduction of media with minimum cytotoxicity such as Puck's fibroblast medium[34] has made it possible to initiate cell cultures with excellent regularity from almost any body tissue, individual, and many mammalian species.

When cells are dissociated from tissue material, profound adaptive processes occur during an initial period (*lag period*), in which certain cells of the explant make the transition from a possibly non-multiplying state (*maintenance state*) in the body to a state of very high reproduction rate *in vitro* (*adaptation*). Often only a small fraction of the explanted cells are capable of initiating reproductive growth in the new *in vitro* environment. As indicated in a case of explanted human skin studied by the author, this fraction may be as small as 10^{-3} to 10^{-4}. In this case approximately 2×10^5 trypsin-dispersed cells extracted from normal skin[40] were cultured with Puck's fibroblast medium in Petri-dishes. Ten days after the seeding of the plates a few fibroblasts appeared as pairs and quadruplets in several isolated areas on the plates. Four weeks later these foci of active growth yielded wellseparated macroscopic colonies. An average of 10 colonies per plate, i.e. per 2×10^5 plated cells, was counted (see Fig. 7). If we assume that every colony originated from a single cell (as is indicated in this case), we obtain for the plating efficiency of these explant-cells a value of about 5×10^{-4}. The majority of cells which remained in lag, did not detach from the glass and could be indentified after many weeks (see Fig. 8). However, a certain portion of the cells which did not divide, stretched out on the glass surface and grew into mononucleate giant cells (see Fig. 9). Upon transplantation the primary colonies of survivors yielded a fast growing cell strain with diploid karyotype which has been maintained now for twelve months. In contrast to this in many other cases reproductive growth is initiated in a large fraction of the explanted cells after a short lag period, and foci of active growth appear in numerous areas after only a few days.

Undoubtedly *selection* processes play a role in many instances once growth is initiated in primary cultures. Since primary cell cultures are heterogenous, selection may operate because of differential reproduction rates, survival rates or migratory capacities of the different phenotypic (morphologic and functional) or genetic (chromosomal) variants which compose primary cell populations.

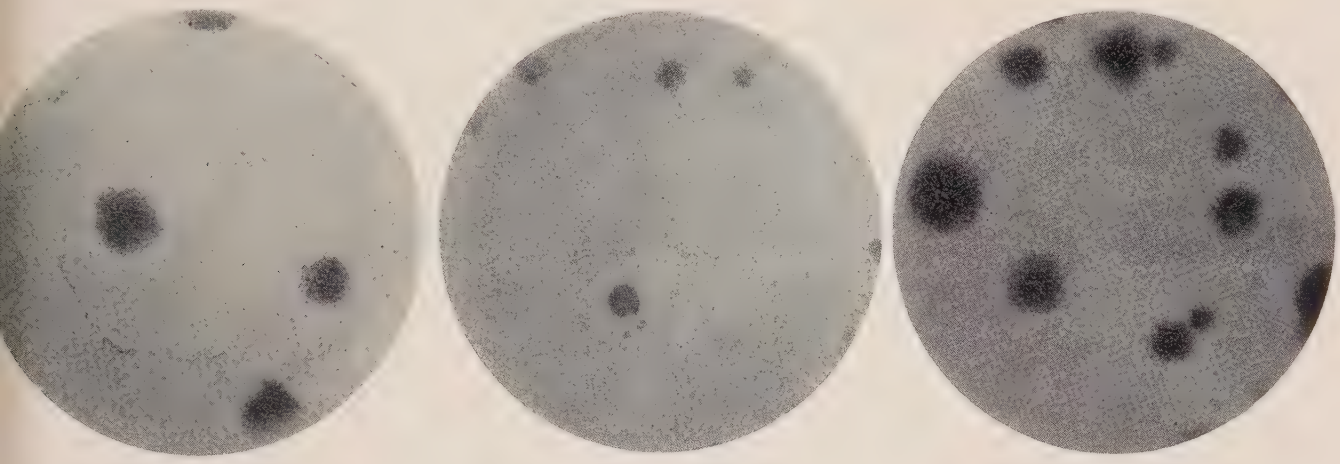

Fig. 7. Primary colonies formed by skin fibroblasts. (Primary culture of trypsinized adult human skin).

Fig. 8. Cells remaining in dormant state (lag) in primary culture of trypsinized adult human skin.

[40] Split thickness skin from an adult human female (caucasian).

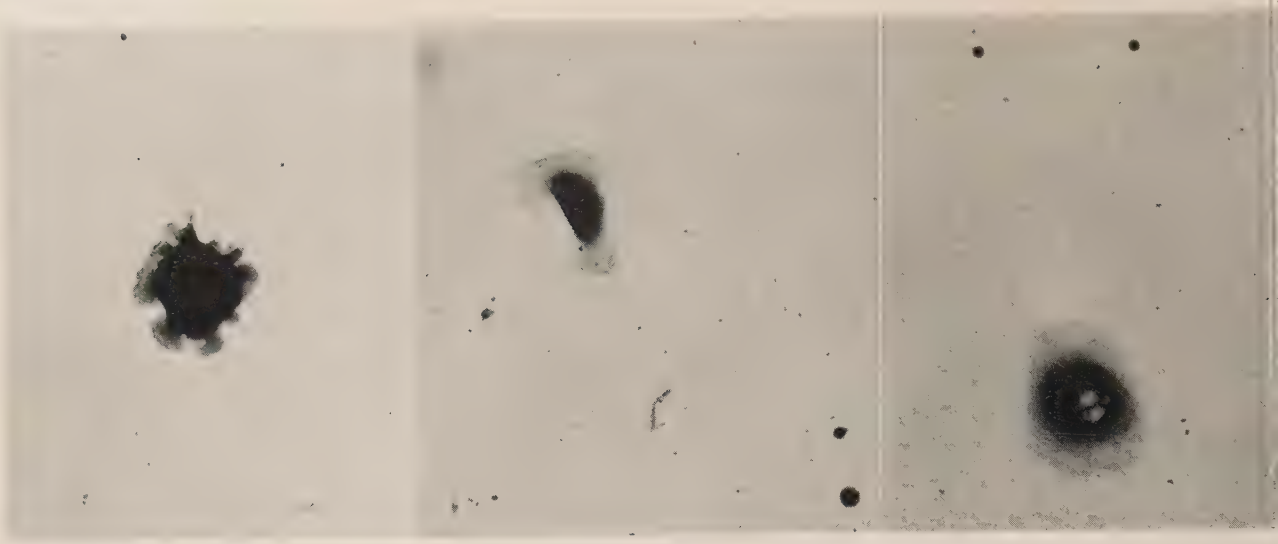

Fig. 9. Giant cells formed in primary culture of trypsinized adult human skin.

Heteroploid-cell transformation. When initiated and subcultured in conventional nutrients (growth media containing adult human, horse or bovine serum supplements), primary cell cultures with diploid karyotype, after several passages (culture transplants), undergo a drastic transformation from euploidy to heteroploidy[41, 42]. During this process alterations of the chromosomes, both in number and morphology, occur spontaneously concurrent usually with a temporary refractory state (non-multiplying phase) of cell reproduction. Shortly before the refractory state, aneuploid cells begin to accumulate in the culture and to replace the original diploid cells. Finally a characteristic heteroploid karyotype dominates in the cell culture which has now attained an unlimited capacity to multiply *in vitro* in the same growth medium as the original (primary) cell culture. In the newly formed cell line (*established heteroploid-cell strains*) cells with normal-diploid karyotype are completely missing and cannot be re-established in these cultures. Thus during the process of transformation the normal diploid karyotype is irreversibly lost. In general heteroploid cell transformation is initiated after about seven passages, i.e. after about two months of culture *in vitro*.

Cultures with stable euploid karyotype. Long-term culture of normal mammalian cells with stable diploid chromosome complement has been accomplished[34] to a large extent in the case of mammalian cells with fibroblast-like morphology mainly by the use of pretested[34] fetal bovine serum instead of adult mammalian serum for incorporation into a defined molecular background medium. In such a growth medium the usual pattern of chromosomal heteroploidy and growth inhibition fails to occur if frequent media changes keep cell nutrition optimal, and if control of pH (7.0–7.1) and temperature (37° C) is strict. The diploid fibroblast-like cells reproduce under these conditions with a generation time of about 16–24 h as do established cell strains, and chromosome analysis of the human cell lines uniformly yield, for 6 to 9 months and probably longer, the characteristic complement of $2n = 46$ (for normal diploid human cells) (see Fig. 10). The incidence of polyploidy and aneuploidy in these long-term cultures is close to or equal to that observed in primary human cultures (2% to 3%).

Evidence obtained by Puck *et al.* indicates that heteroploid-cell transformation and concurrent non-multiplying episodes occurring in cells grown in conventional growth media are artificially induced *in vitro* by mitotic inhibitors present in adult mammalian sera. Adult mammalian sera contain a large amount of γ-globulin while fetal bovine serum is deficient in this protein fraction (see Fig. 11). Preliminary experiments[34] indicate that the γ-globulin fraction of adult serum proteins contains factors toxic to cell growth *in vitro*. These growth inhibitors present in adult sera (but not in fetal bovine serum) may be the major cause for the induction in basically diploid cell populations of multipolar spindel formation and other alterations of the normal course of mitosis which give rise to numerous polyploid and unbalanced aneuploid karyotypes (refractory state of growth), and lead to the final overgrowth of a heteroploid cell type with superior selective advantage (established altered cell strain). Thus experimental evidence indicates that heteroploid cell transformation *in vitro* is an artefact induced in the conventional suboptimal culture environment, and that normal (diploid) cells have basically a stable chromosomal constitution[43].

[41] T. C. Hsu, C. M. Pomerat, and P. S. Moorhead, J. nat. Cancer Inst. *19*, 867 (1957).

[42] P. B. Medawar, Biol. Rev. *22*, 360 (1947).

[43] Tumor cells may not have such stable properties.

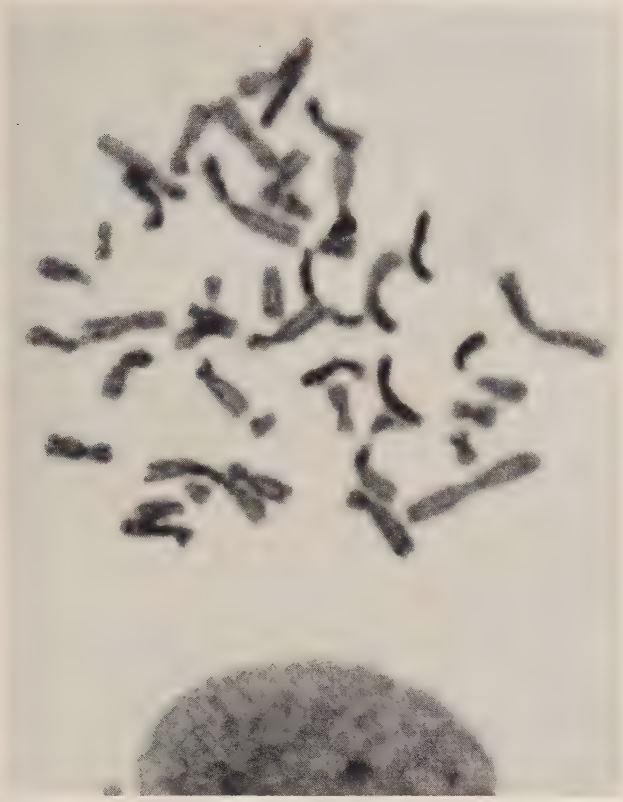

Fig. 10. Normal-diploid human chromosome complement (female), $2n = 46$, of fibroblast-like cells maintained for 6 months in culture. (Strain M-1).

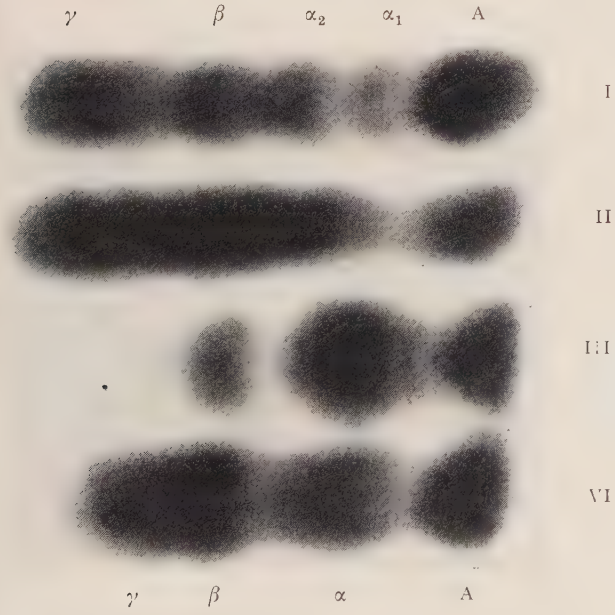

Fig. 11. Paper electropherograms of mammalian sera.
I: adult human serum. II: adult horse serum. III: fetal bovine serum. IV: adult bovine serum, i.e. calf serum. A: Albumin. α: α-globulin. β: β-globulin. γ: γ-globulin.

The possibility to maintain in long term culture mammalian cell strains with normal and stable chromosome constitution represents a major achievement which permits new approaches to fundamental problems of somatic cell variation and new uses of cell cultures for practical purposes. The elimination of the chromosomal alterations and the concurrent refractory state of growth permits systematic studies in somatic cell genetics or of the growth and metabolism of normal and malignant cells. Moreover, it may prove to be one of the keys to the establishment of functional-cell banks for therapeutic applications (bone marrow-cell banks, skin-cell banks) and for other uses (*in vitro* production of antibodies, hormones, and viruses).

The Requirements for Growth of Single Human Cells in vitro

The studies of Eagle[8,9] and of others show clearly that both altered (heteroploid) and normal (diploid) human cells in serially propagated monolayer culture require for growth a minimum of 12 amino acids (L-arginine, L-cystine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, β-phenyl-L-alanine, L-threonine, L-tryptophane, L-tyrosine, L-valine), one half amide, L-glutamine, 9 vitamins (choline, folic acid, nicotinamide, pantothenic acid, pyridoxal, riboflavin, thiamine, biotin, i-inositol), five electrolyte ions (Na^+, K^+, Ca^{++}, Mg^{++}, $H_2PO_4^-$) and glucose (*minimum essential molecular growth factors*). In a minimal medium composed of these essential molecules and supplemented with a small amount of dialyzed mammalian serum (Eagle's *basal growth medium*) a variety of human cell strains can be propagated indefinitely and with a generation time in the logarithmic growth phase approximating 20 to 24 h. In this minimal medium the omission of any single molecular growth factor leads to early death of the whole cell culture.

Additional nutritional requirements for growth, however, manifest themselves not only in different human cell strains but also under modified conditions of culture. Thus normal (diploid) human cells with fibroblast-like morphology have additional not yet clearly defined molecular requirements for sustained growth in dense monolayer cultures[34,35], on the other hand altered epithelial-like cells which multiply vigorously in heavily inoculated monolayer cultures and in basal growth medium fail to grow in the same minimal medium when the cultures are initiated with a small number of single cells.

The specific growth factors for single human cells have been determined in various altered human cell strains. Studies by the author are in progress to determine these factors in normal (diploid) human cells of epithelial-like morphology.

1. The effect of dialyzed serum on single Detroit-98C6b[44] *cells.* Heavily seeded static monolayer cultures of this cell line proliferate with a doubling time of about 24 h in the basal mixture of Eagle supplemented with 10% of serum which has been dialyzed for 24 h against running saline at 4°C (basal growth medium). Attempts, however, to clone single cells according to the method of Puck *et al.*[32, 33, 45] in the same growth medium, fail. In basal growth medium the rate and extent of cell multiplication decreases in proportion to the number of cells plated ('Dilution'-effect[32]). In the presence of dialized serum the cells attach to the glass surface but the majority of cells undergo only a limited number of divisions, forming abortive colonies, when plated out in small numbers (10^2 to 10^3 cells/10 ml medium).

Dialysis removes from serum the specific molecules essential for sustained growth of widely separated single cells but not essential for closely packed cells. Figure 12 illustrates the activity of serum as a function of time of dialysis. In these experiments fixed volumes of serum were dialyzed against 20 vol each of saline (6.8 g NaCl/l) at 7°C with agitation. Under these conditions the activity of serum decreases by approximately 70–80% during the first 30 min of dialysis, indicating that small or medium-sized molecules are composing the active molecular fraction of serum.

2. The effect of serum ultra-filtrate on single Detroit-98C6b cells. Heat treatment of adult horse serum ultrafiltrate results in the formation of a precipitate. Addition of the supernatant (protein free) ultrafiltrate fraction to basal growth medium restores the plating efficiency of single Detroit-98C6b cells to the value obtained in medium containing whole serum. Thus the active factors of serum are heat-stable molecules.

3. The 'feeder' effects of dense Detroit-98C6b cultures on single cells. Feeder-layer experiments carried out in basal growth medium demonstrate that actively growing cells in dense monolayer culture synthesize and release into the supernatant minimal nutrient the specific molecules necessary for sustained growth of widely separated cells in the same minimal medium[45].

4. The effects of defined molecules involved in the tricarboxylic acid cycle on single Detroit-98C6b cells. Pyruvate is an important metabolite involved in the TCA cycle and in the anaerobic conversion of glucose to lactate. Supplementation of the basal growth medium with sodium pyruvate at a concentration of 1 *mM*/l increases the plating efficiency in the average to about one-half of the standard value. In the presence of ATP (1 mg/l) or vitamin B_{12} (0.1 mg/l) the effect of pyruvate is reduced. Further supplementation with citric acid (30 mg/l), succinic acid (2 mg/l), lactic acid (100 mg/l), fumaric acid (2 mg/l) does not increase the effect of sodium pyruvate (1 *mM*/l) on the plating efficiency. Oxalacetic acid, another metabolite of the TCA cycle, does not show any activity in the presence of pyruvate.

5. The effects of nonessential amino acids and of pyruvate on single Detroit-98C6b cells. In cell strain HeLa and HeLa-S3 supplementation of the minimal medium (containing dialyzed serum) with the non-essential amino acid L-serine suffices to augment the plating efficiency of single cells to its standard value in medium containing whole serum[33]. In contrast to HeLa cells the plating efficiency of Detroit-98C6b cells is increased only to about one third of the standard value when the basal growth medium is supplemented with the seven non-essential amino acids L-alanine, L-aspartic acid, L-asparagine, L-glycine, L-glutamic acid, L-proline, and L-serine at concentrations of 0.2 *mM*/l. However, the addition to the basal growth medium of sodium pyruvate (1 *mM*/l) in combination with the seven non-essential amino acids or with L-serine alone at 0.2 *mM*/l yields plating efficiencies close to or in excess of the standard value and fully developed macroscopic clones (see Fig. 13 and 14).

6. Conclusions. Human cell strains have a basic and natural requirement for growth consisting of 12 amino acids, 1 half amide, 9 vitamins, 5 electrolytes, and glucose. These are the *minimum essential molecular growth factors.* They initiate, in the presence of serum protein, sustained growth of a variety of cell strains in heavily inoculated monolayer cultures. Widely separated single cells in small inocula of these cell strains have additional molecular requirements for sustained growth *in vitro* such as non-essential amino acids or pyruvate, depending upon the cell strain. These 'anomalous' requirements of small inocula—*which we define as quasi-essential growth factors*—are either (a) metabolites easily lost from the intracellular pool; (b) compounds which facilitate the biosynthesis of certain

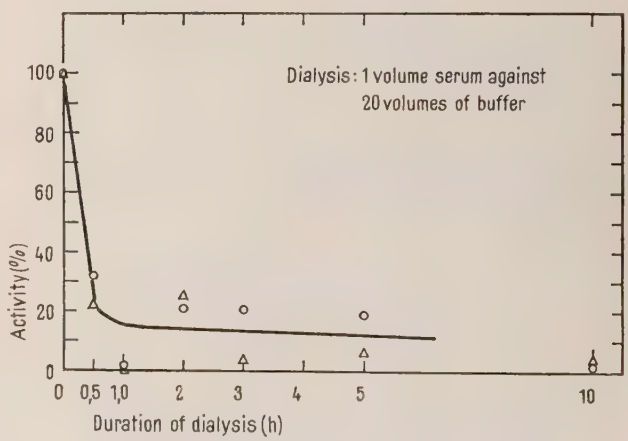

Fig. 12. Activity of dialyzed serum in promoting growth of single Detroit-98C6b cells as a function of duration of serum dialysis.

[44] A clonal derivative of the Detroit-98 strain (modified human sternal marrow).

[45] H. Moser and C. Madden Annual Report of the Biological Laboratory, Long Island Biological Association, Cold Spring Harbor, L. I., N. Y., 69th year (1959).

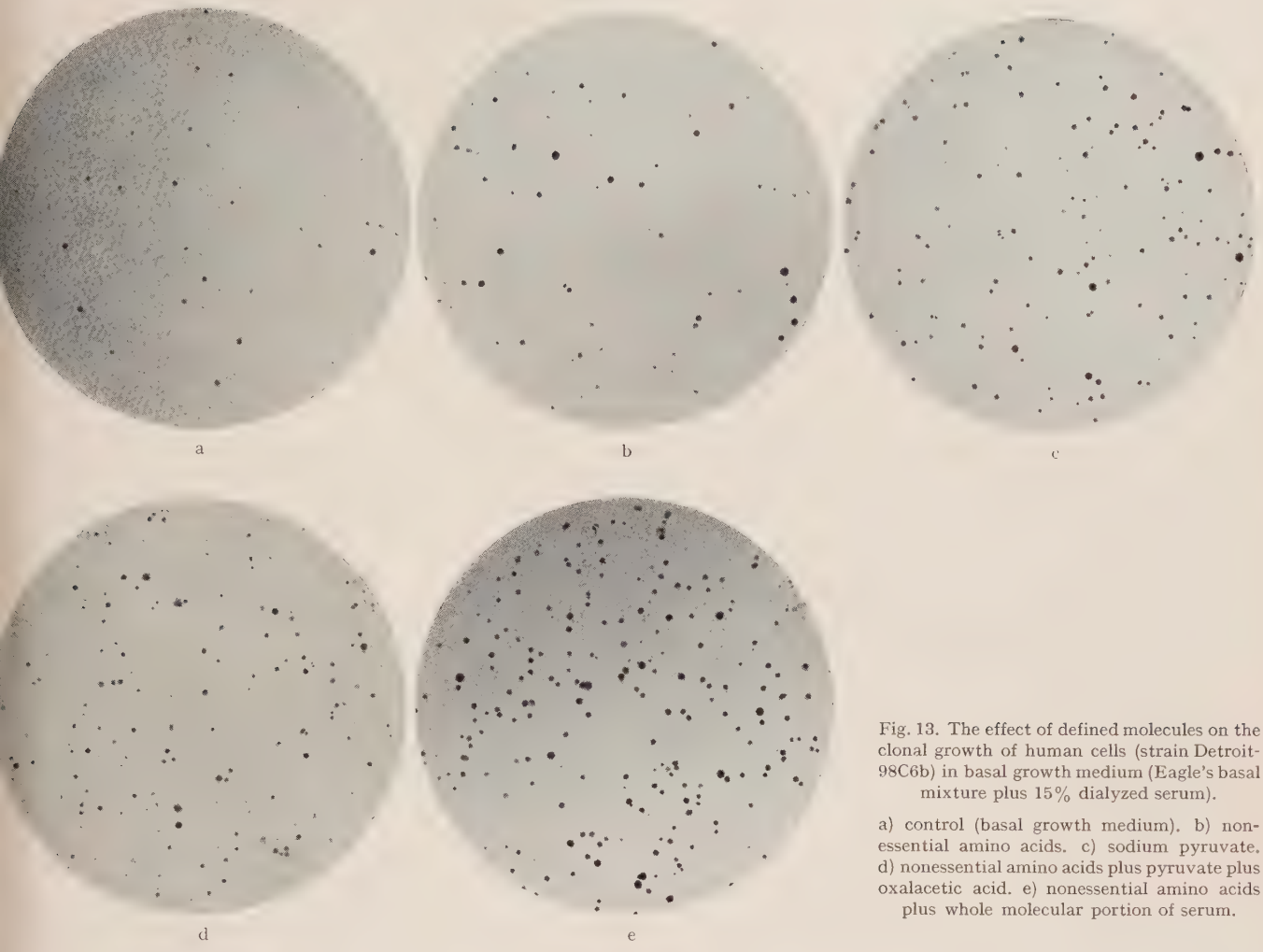

Fig. 13. The effect of defined molecules on the clonal growth of human cells (strain Detroit-98C6b) in basal growth medium (Eagle's basal mixture plus 15% dialyzed serum).

a) control (basal growth medium). b) nonessential amino acids. c) sodium pyruvate. d) nonessential amino acids plus pyruvate plus oxalacetic acid. e) nonessential amino acids plus whole molecular portion of serum.

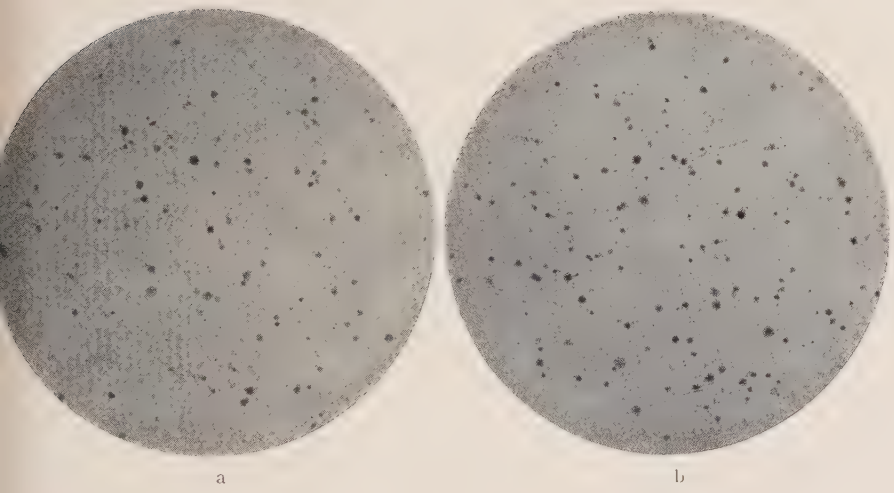

Fig. 14. The effect of defined molecules on the clonal growth of human cells (strain Detroit-98C6b) in basal growth medium.

a) control (standard cloning medium, i. e. Eagle's basal mixture plus nonessential amino acids plus 15% whole serum). b) Eagle's basal mixture plus 15% dialyzed serum plus L-serine plus sodium pyruvate.

metabolites; (c) compounds which increase the retention of metabolites by the cell, or (d) compounds which inhibit cytotoxic materials (such as proteolytic enzymes) excreted by widely isolated cells. These molecular quasi-essential growth factors or their active substitutes are provided by complete serum and lost upon serum dialysis.

The nature of the supplements to the basal growth medium which are active in promoting sustained growth of single somatic cells suggests that they are metabolites, and they or their precursors are either easily lost from single cells or have a small biosynthetic rate. Let us assume (see Fig. 15) that B is a compound which is synthesized from an essential amino acid (sup-

plied externally), A, at rate k_1, and is converted into C, a building block of proteins and of cell structure, at rate k_3. If B leaks from the cell into the external medium at rate k_2, synthesis of C and therefore of cell growth and multiplication is accomplished only if the biosynthetic rate k_1 exceeds the rate of removal of B from the intracellular pool into the external medium. The rate of leakage of B (per cell) is obviously a function of the surface area (per cell) exposed to the medium, and of the rate of accumulation of B in the external medium. Because the free surface area per cell in monolayer cultures decreases with increasing cell density—due to cell reaggregation and packing of cells—and because the accumulation rate of B in the media increases with increasing cell density, the rate of leakage increases with decreasing cell density in the inocula in a medium lacking B ('dilution' effect). However, if B is supplied with the nutrient fluid, leakage of B is reduced or suppressed because of concentration effects, and the intracellular B pool maintains a level adequate for continued synthesis of C and therefore for sustained growth of isolated single cells.

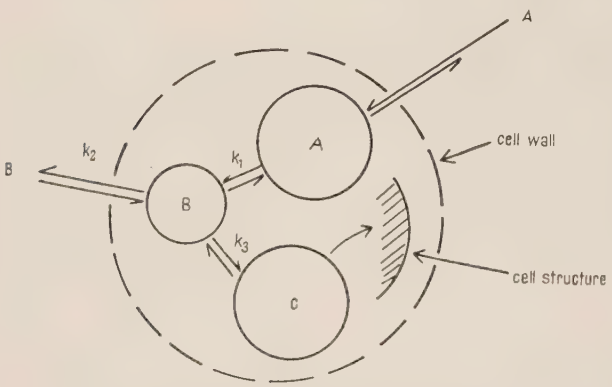

Fig. 15. Model of isolated mammalian cell.

Studies of the 'anomalous' nutritional requirements lead to the formulation of *minimal media for single mammalian cells*. The development of such growth media is stimulated particularly by their possible use for rapid selection of rare biochemical mutants in large cell populations ('selective media' for mammalian cells).

The Lethal and Semi-Lethal Effects of Radiations on Human Cells in Culture

The methods for rapid growth of single cells into well separated macroscopic colonies developed by Puck *et al.* made possible a quantitative assessment of the lethal and semi-lethal effects of radiation on human cells under controlled environmental conditions. The effects of both ionizing radiation[46–48] and of ultraviolet light[32, 48, 49], have been studied in this fashion in a variety of human somatic cell strains.

1. The fate of cells irradiated with X-rays or ultraviolet light. The irradiation of plates seeded with singel mammalian cells destroys, in a fraction of the cell population (specific survival fraction) the capacity of a single cell to grow into a macroscopic colony (composed of from 10^3 to 10^4 cells). The fate of irradiated cells that fail to form visible colonies is the following: An irradiated cell may form an *abortive colony*, that is, it may undergo a limited number of cell divisions to form a self-sustaining microcolony of less than 50 cells. An irradiated cell may fail to divide at all, simply disintegrate without leaving a trace on the stained plate, or grow into a single *giant cell* (see Fig. 16). The fraction of giant cells increases with increasing radiation dose. Exposure of a plate seeded with about 10^4 HeLa cells to an X-ray dose of 1000–5000 r will, after incubation for two weeks yield a population consisting entirely of giant cells[46]. Giant cells may survive for long periods without regaining their capacity to divide even in regularly renewed nutrient, and are highly susceptible to infection and destruction by virus[46]. Observations by the author indicate that X-rays induce a higher proportion of giant cells and abortive colonies than does a corresponding dose[50] of ultraviolet radiation.

2. Ultraviolet survival curves. By application of the plating methods described earlier, and with further modification of those techniques Moser and Tomizawa[32] determined the survival curves of ultraviolet treated cells of the following hyperploid epithelial-type cell strains: Detroit-98 (derived from human adult sternal marrow), Detroit 98C6b (clonal derivative of Detroit-98), D-189 (malignant human foreskin of Leighton), Hayflick-Fernandez strain derived from human amnion. Figures 17, 18, 19 represent curves in which the specific survival fraction[51], S_D, is plotted on a logarithmic scale against the incident dose of ultraviolet light, measured in mW-sec cm^{-2}. (1 mW-sec cm^{-2} = 100 ergs mm^{-2}).

When plotted in this fashion, the UV-survival curves of single heteroploid somatic cells exhibit two distinct sections, a log-linear portion with an initial shoulder, and a non-log-linear section manifesting itself at high UV doses. The existence of an initial shoulder in all the UV-survival curves indicates a multiple-hit-killing mechanism of UV irradiation in heteroploid human somatic cells. The first portion of the survival curves fits the equation $S_D = 1 - (1\text{-exp}\ (-\ kD))^n$ where D is the

[46] T. T. Puck and P. I. Marcus, J. exp. Med. *103*, 653 (1956).
[47] T. T. Puck, D. Morkovin, P. I. Marcus, and S. J. Cieciura, J. exp. Med. *106*, 485 (1957).
[48] T. T. Puck, Amer. Nat. *94*, 95 (1960).
[49] H. H. Lee and T. T. Puck, Radiation Res. *9*, 142 (1958).
[50] I. e. a dose that produces an equal fraction of colonyforming survivors.
[51] If $S(0)$ stands for the survival fraction at dose $D = 0$ (controls), $S(D)$ for the survival fraction at dose D, the specific survival fraction for D is defined as $S_D = S(D)/S(0)$.

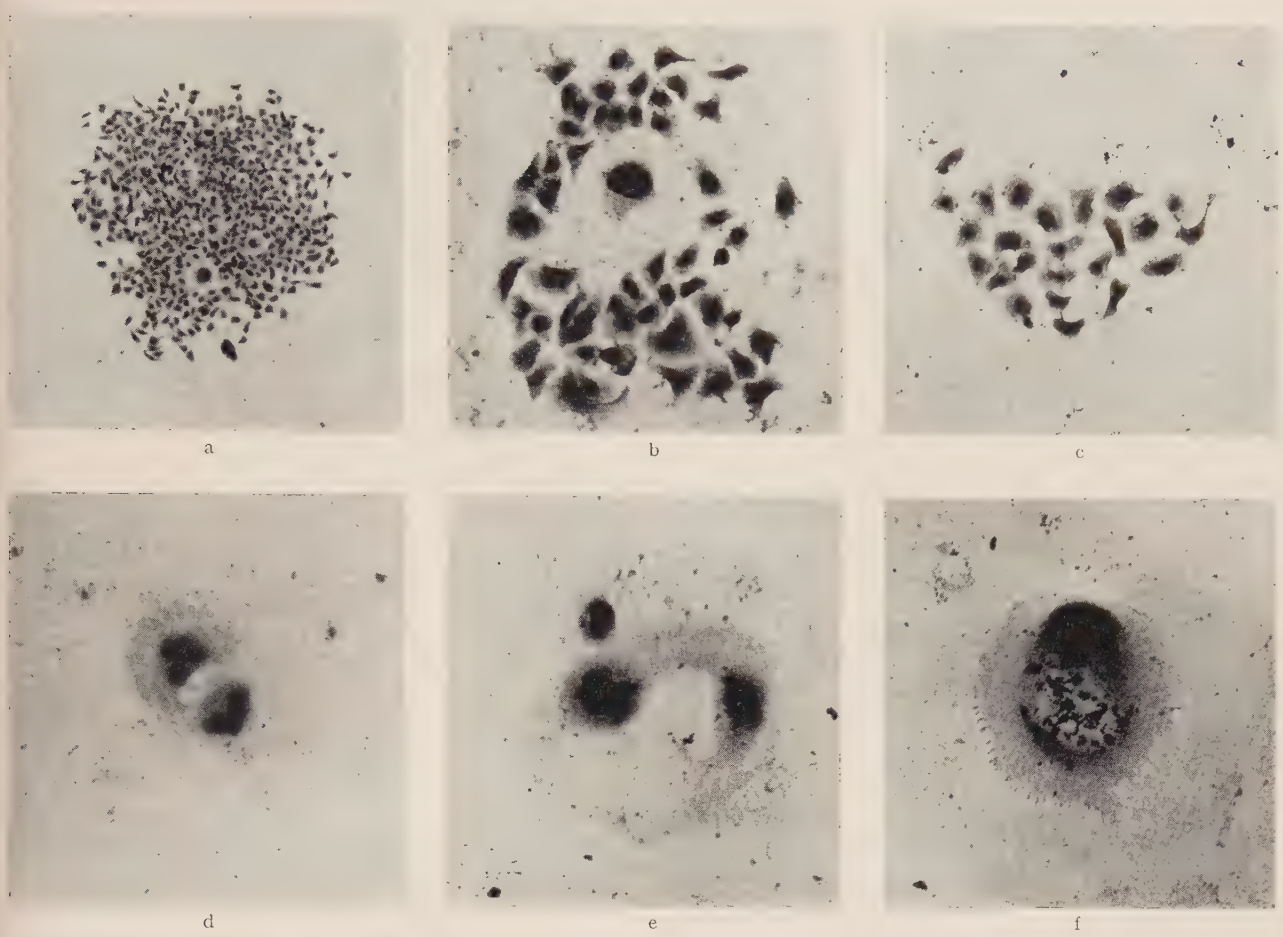

Fig. 16. Fates of ultraviolet irradiated epithelial-like human cells (altered-heteroploid strain Detroit-98C6b).

a) macroscopic colony formed by UV survivor. b) abortive colony with single giant cell. c) abortive colony without giant cell. d, e) multinucleate giant cells. f, g) mononucleate giant cells.

Tab. II. Parameters of UV Survival Curves.

Strain	n	D_0 (mW-sec cm^{-2})	D_x (mW-sec cm^{-2})	S_x
D-189	2.5	0.775	5.94	1.0×10^{-3}
Human Amnion	1.5	1.070	4.95	1.4×10^{-2}
Detroit-98	8.5	0.684	5.40	3.0×10^{-3}
Detroit-98C6b (clonal strain)	3.0	1.000	4.95	2.0×10^{-2}

UV dose, k a constant measuring the radiation sensitivity, and n the hit number. The hit number is determined by extrapolation of the straight line of the log-linear portion of the curve to its intercept of the S_D axis, $S_0{}^* = n$. The value of k is determined from the slope of the log-linear portion of the curve. The constant k is related to the mean lethal dose, D_0, i.e. the dose which reduces, in the long-linear portion of the curve, the survival fraction to e^{-1} (or 37%).

Our analysis of the UV survival curves in hyperploid human cells yields hit numbers varying from 1.5 to 8.8 and mean lethal doses, D_0, varying from 78 to 107 ergs mm^{-2} (Table II). At a critical UV dose, D_x, which corresponds to a specific survival fraction, S_x, the radiation sensitivity begins to change as a function of UV dose. The dose at which radiation resistance 'develops' in UV-irradiated heteroploid somatic cells depends on the cell strain tested, and varies in our case from 500 to 600 ergs mm^{-2}.

Although the survival curves as such lend themselves only to a limited understanding of the UV-killing mechanism, the strain-specifity of their parameter values indicates the existence of differences in the genetic apparatus of the cells belonging to different heteroploid

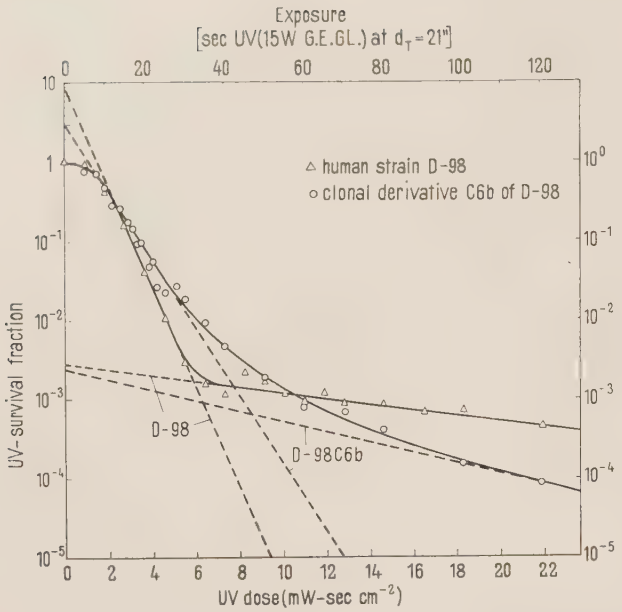

Fig. 17. Ultraviolet survival curves of strains Detroit-98 and of clonal derivative Detroit-98C6b.

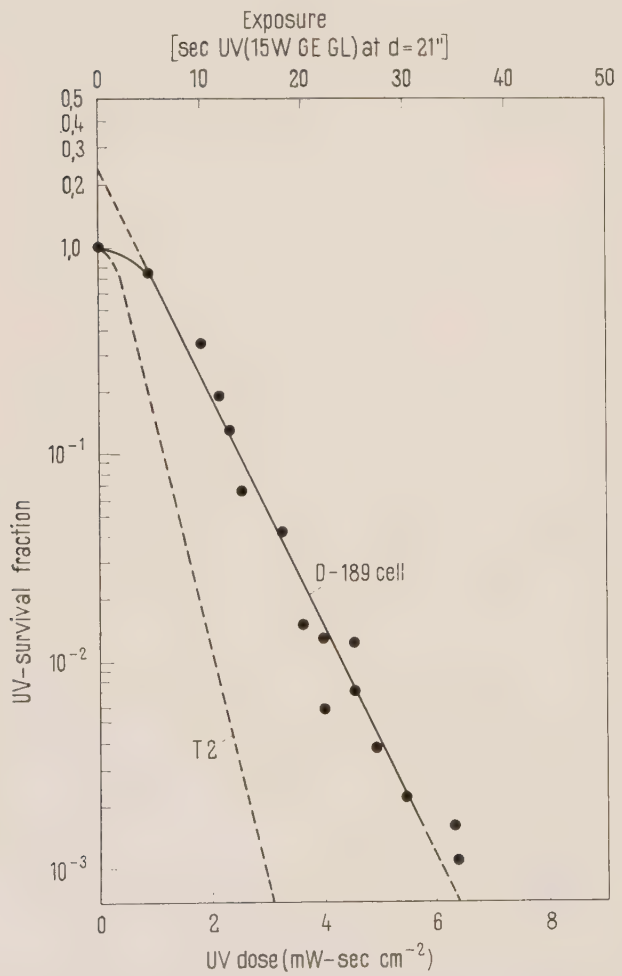

Fig. 18. Ultraviolet survival curve of human strain D-189 and of bacteriophage T2.

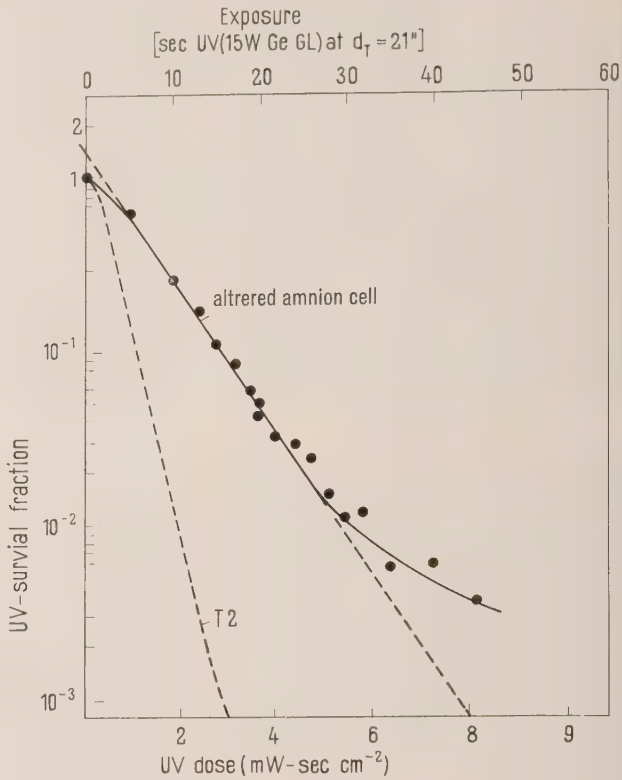

Fig. 19. Ultraviolet survival curve of human amnion-cell strain Hayflick-Fernandez and of bacteriophage T2.

strains. Cytological observations support this conclusion.

X-ray survival curves. The studies of Puck *et al.*[46–48] demonstrate that also X-ray treated human cells *in vitro* display multi-hit type survival curves. While the hyperploid cells yield hit numbers close to 2 the diploid-fibroblast-like cells have a hit number close to one. The mean lethal dose which is about 100 r for the altered heteroploid cells and only about 50 r for the diploid cells indicates the 'protective' effect of supernumery chromosomes on the reproductive death of human cells which have been exposed to ionizing radiation.

Acknowledgments. The author is indebted to Dr. S. Graff, Professor of Biochemistry, for critical review of the manuscript, and to Mrs. Ada Graff and her staff for the preparation of the paper electropherograms presented in this publication. For the photographic work the author wishes to thank Mr. E. R. Hajjar of Columbia University, Mr. H. Jones of the Carnegie Institution of Washington (Fig. 16) and Dr. M. K. Dutt of the Carnegie Institution of Washington (Fig. 10).

Zusammenfassung

Der Verfasser gibt eine kurze Übersicht über die neueren Methoden der *in vitro*-Kultur von Säugerzellen. Eingehend wird die Entwicklung von stabilen Kulturen diploider Zellen und Untersuchungen über die spezifischen Wachstumsfaktoren isolierter menschlicher Zellen besprochen. Auch die Wirkung der Ultraviolettstrahlung auf solche Zellen wird diskutiert.

Studies on the Active Center of Monoamine Oxidase

E. A. Zeller*

At the occasion of the 500th anniversary of the University of Basel it is altogether fitting that we who went here through the first steps of our academic career should ponder about our debts to this institution. Compared with many universities in other countries, ours is a small one indeed and yet, how many brilliant ideas were born here, ideas strong enough to change man's thinking. Even if we concentrate on biochemistry alone, what an array of famous names arises in our minds, from C. F. Schönbein to F. Miescher, G. von Bunge, and T. Reichstein. The two department heads who opened the door to biochemistry for me were associated for a long time with some of the oldest and most outstanding institutes in this field: before coming to Basel, Karl Spiro worked for almost 25 years with F. Hofmeister in Strassburg, and Siegfried Edlbacher studied most successfully under A. Kossel in Heidelberg. Thus several lines of classical tradition ran within or led to the Department of Physiological Chemistry and we, the younger members of its staff, could in all our pathetic lack of experience not help but feel 'als der Erbe aller Vornehmheit alles vergangenen Geistes und der verpflichtete Erbe', to use the inspired words of F. Nietzsche, another teacher at our *alma mater*[1].

It was in S. Edlbacher's laboratory that I first became acquainted with the enzymology of amino acid and amine metabolism[2]. I also received a helping hand from M. Guggenheim, the author of the classical text on *Die biogenen Amine*. Ever since and with the help of a succession of more than twenty able assistants, I have spent more time and energy on this topic than on any other problem.

Active Centers of Enzymes

Before we approach our subject we have to define the concept of the active center which 'is that special part of the enzyme protein structure which combines with the substrate'[3]. This elegant definition is not always sufficient as the following example will demonstrate: when an aliphatic amine reacts with monoamine oxidase (MO) the amino group as well as the aliphatic chain participate in forming the operative complex with the active center. However, if – in β-position – we introduce a benzene ring, a hydroxy or a second amino group, the new residues also contribute to the binding of these substances to the MO[4]. We must, therefore, differentiate between the primary active center which is always involved in the formation of the enzyme-substrate complex and, around it, a secondary area which only in certain cases establishes bonds with the substrate (Fig.).

The elucidation of the structure of active centers of enzymes is one of the most important, albeit most formidable tasks of modern enzymology. Various ways toward this goal can be chosen. We may determine the amino acid sequence of the active center, which has been marked by a prosthetic group or by substrate or inhibitor molecules[5]. While this procedure often leaves some doubt about the nature and extent of the active center, another approach leads – by definition – directly and unambiguously to the desired enzyme part. It consists of offering a large number of suitably chosen substrates and closely related inhibitors to a given enzyme and deriving, from the kinetic data, affinities and other information. From all these substances we pick out those which fit best into the slot of the active center. Thus gradually a pattern of its structure will emerge. We used mainly this latter procedure in our studies.

Primary and Secondary Area of Active Centers

* Department of Biochemistry, Northwestern University Medical School, Chicago, Ill.

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Oxidative Deamination

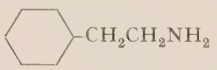
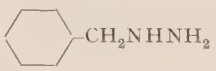
In order to illustrate the concept of active centers we turn to a group of enzymes involved in the oxidative deamination of amines and amino acids. The overall reaction catalyzed by all these enzymes – with some justifiable simplifications – can be summarized by equation I. This apparent similarity

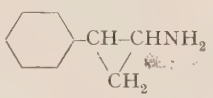
$$\text{I} \quad RR'CHNH_2 + O_2 + H_2O = RR'CO + NH_3 + H_2O_2 \qquad (R' = \text{H or COOH})$$

between the enzymes of this group may not be a mere formal one but could reflect a similarity in action mechanisms. If this were true the actual differences between various amino acid oxidases, monoamine oxidases and diamine oxidases would have to be found in the first steps of the process of the enzyme-substrate interaction. During this phase the substrate molecule is arranged on the active surface in such a manner that the *hydrogen donating* group of the substrate goes into the closest proximity and into the proper spatial relationship to the *hydrogen accepting* residue of the enzyme. It is this sequence of events and the forces directing it which differ from one oxidatively deaminating enzyme to another. – From here on this discussion will be limited to a presentation of MO data.

Substrate Patterns of Monoamine Oxidase

A series of phenylalkylamines were synthesized and tested against various MO preparations[6,7]. Phenylethylamine (Formula II), the compound with the two-membered aliphatic chain, turned out to be the best substrate, both with respect to the affinity (K_M) and reaction rate (V_{max}). When the aromatic ring was left out, the resulting ethylamine was only weakly attacked[8]. Obviously, the amino group, the aliphatic chain, and the benzene ring all are of importance to the formation of the enzyme-substrate complex.

II  III 

IV 

Phenylcycloalkylamines

Since competitive inhibitors have to fulfill fewer conditions for interacting with the enzyme molecule than substrates, it is often relatively simple, with their help, to obtain valuable information about the structure of active centers. As an example the *cis*- and *trans*-phenylcyclopropylamines (see Formula IV) are mentioned[9]. These substances do not qualify as substrates of MO, but they block this enzyme more strongly than any other compound[10–12]. At 10^{-7} M concentration, half of the MO is inhibited ($pI_{50} = 7$). When the benzene ring is left out or when the structure is arranged in a slightly different manner the inactivating power drops tremendously: cyclopropylamine is almost inactive ($pI_{50} = 1{\cdot}5$) and 1-phenyl-cyclopropylamine displays a much smaller efficiency than the 2-phenylcyclopropylamine ($pI_{50} = 5{\cdot}1$).

Inhibition of Monoamine Oxidase by Iproniazid[13]

After the discovery of the remarkable inhibitory power of iproniazid for MO[14–17], one of our principal tasks was to find out whether this compound attacked MO at the same spot as the substrates did. The phenomenon of competitive inhibition[17–18] pointed clearly toward the active center as the primary target of iproniazid. The observations convinced us that iproniazid (V) could become a precious tool for the exploration of MO. Therefore, we took this molecule apart and tested its components and related structures on MO[6,19,20]. After having studied a great number of substances it became obvious that the effect of iproniazid on MO was due to the isopropylhydrazine residue (see bold printed part of the iproniazid molecule V) in much the same way as in the classical work of Tréfouel, Bovet, and Nitti, the sulfanilamide residue was recognized as the essential part of Prontosil.

V

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Phenylalkylhydrazines

Experiments carried out with iproniazid induced us to test alkylhydrazines and phenylalkylhydrazines which turned out to be excellent MO-blocking agents. Some data obtained with a series of phenyl-*n*-alkylhydrazines ($C_6H_5(CH_2)_nNHNH_2$, $n = 0$ to 3), may serve for illustration. In this series benzylhydrazine ($n = 1$, see Formula III) was the most effective agent, closely followed by phenylethylhydrazine ($n = 2$)[6,7]. In addition to benzylhydrazine, some analogous substances such as 2-thiophene-methylhydrazine and 3-pyridine-methylhydrazine inhibited MO more strongly than most of the hydrazine derivatives[7].

Homologous Enzymes

When data on substrate and inhibitor patterns are discussed one has to keep in mind that they are valid only for a given species or organ because the enzymic response to various substances changes with the source from which the enzyme was prepared. This is certainly true for MO[8,21–23]. One example has to suffice: rabbit liver MO is much less sensitive ($pI_{50} = 5{\cdot}7$) toward the phenylcyclopropylamines than beef liver MO[10]. The difference cannot be accounted for by differences in the accessibility of MO in the two kinds of mitochondria since solubilized rabbit liver MO displayed the same behaviour as that of whole mitochondria. Following the terminology of comparative anatomy it has been suggested that a group of closely related, but not identical enzymes, be called homologous[24,25]. The chemical differences between homologous enzymes may pertain to amino acids away from the active center, as seems the case for sheep and beef pancreas ribonuclease[26], or within the active center, as some data mentioned above and related informations about MO suggest.

Active Center of Monoamine Oxidase

All three outstanding agents – phenylethylamine II, benzylhydrazine III, and phenylcyclopropylamine IV – display the same backbone: a benzene ring, a two-membered side chain, and an amino group. When the benzene ring is left out, the substrate or inhibitor activity drops sharply or disappears almost entirely. When we remember how closely related CH_2- and NH-residues are – as expressed by Grimm's theorem of the hydride shift (Hydridverschiebungssatz) and demonstrated with many striking examples by Erlenmeyer[27] – the similarity becomes even greater. One can, therefore, hardly avoid the idea that all classes of compounds considered here, amines as well as pseudoamines, act in a similar fashion with MO. Furthermore, the active center of this enzyme may contain a structure complementary to the amines and hence may be made up by an aromatic nucleus and a two-membered side chain[28]. Between the related structures of the substrate of inhibitor molecules and of the active center hydrophobic forces may come into play and help to adjust the smaller molecules to the enzyme surface.

Our results with α-substituted aliphatic amines and hydrazines can be interpreted by the assumption that *one* α-hydrogen is essential for establishing the enzyme-substrate complex or enzyme-inhibitor complex, while the *second* α-hydrogen—if one is available—participates in the process of dehydrogenation. One of several possibilities consists of assigning to one part of the active center the following structure, $- X\text{–}\ddot{Y} -$, in which X serves as an acceptor for the α-carbon, and $\ddot{Y}$ for a proton (derived from an α-hydrogen)[10].

Many other properties of the active center of amine oxidases have been studied with the help of specifically selected substrates and inhibitors in this and other laboratories, but we must limit ourselves here to two conclusions: the active center of these enzymes seems to extend over several *Angstrom* units[29] and its structure appears to be asymmetrical[23,30–32] possibly because it is built up in part by L-amino acids[30].

In vivo Experiments

Shortly after noticing the strong blocking power of iproniazid on MO *in vitro* we observed the equally effective *in vivo* inhibition of brain and liver MO by this drug [15–17]. It took several days before the enzymic activity recovered[17,33], long after all free iproniazid had disappeared from the animal[34]. Similar results were obtained with various species[35] and related inhibitors, including phenylcyclopropylamines[10].

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MO-Inhibitors as Tools in Metabolic, Pharmacological, and Clinical Research

One may ask whether MO and in particular its active center are more than a plaything for the enzymologist. With the following remarks—by necessity extremely brief[36]—it is attempted to answer this question.

Since iproniazid and similarly effective compounds block MO substantially *in vivo* they possibly could alter the metabolism of those biogenic amines which are substrates of MO. This has been shown to be true for the first time by Schayer at Northwestern University who was able to increase the urinary excretion of labelled tyramine in iproniazid pretreated rats[37]. Others demonstrated that, in brain, heart, urine, and other materials, the level of various amines such as serotonin and catecholamines goes up after blocking MO[35, 38, 39]. Obviously, these highly active compounds are protected from being destroyed by MO in much the same way as Loewi prevented acetylcholine by means of eserine[40] from being hydrolyzed by cholinesterase.

The enzymic and metabolic studies described here have thrown an entirely new light on the pharmacology of iproniazid and related drugs which may primarily act by modifying the metabolism of certain biogenic amines. The first experiments confirming this idea were carried out in the Departments of Biochemistry, of Pharmacology—C. A. Dragstedt, E. C. Griesemer, J. A. Wells—and of Medicine—S. M. Feinberg, J. Rebhun—of Northwestern University. We showed that the effect of tyramine on the nictitating membrane of the cat[41] and on the body temperature of the guinea pig [42] was strongly potentiated by iproniazid. Since then a large number of investigations have been performed first by Brodie and then by other outstanding pharmacologists. Among other things they contributed to a better understanding of the pharmacology of reserpine[35, 36, 39].

By now an impressively large body of informations has been accumulated, compatible with the concept that the active center of MO is one of the targets—if not the main one—of iproniazid. This drug was first introduced as an antituberculosum but was subsequently rejected because of some undesirable effects[43]. In two independent experiments the influence of iproniazid on psychotic patient was investigated, this time based on the MO-blocking concept. One study was conducted at the Rockland State Hospital, Orangeburg, New York, by Kline *et al.*[44] and the other at the Veterans Administration Hospital in Hines (Illinois)—Bernsohn, Inskip, Lauer—and at Northwestern University[44]. Today probably more than a million patients suffering of psychoses and other diseases have been treated by 'psychic energizers'.

It is beyond the scope of this paper to evaluate the role of MO in the many therapeutic applications of the so-called MO-inhibitors or to discuss the data presented at national and international meetings which have been devoted in part or *in toto* to the monoamine oxidase inhibitors[36].

Thus a large tree has grown in the rich soil of the 'New World' from small seeds sown at the University of Basel and the concepts and methods of theoretical enzymology have become handy tools for many segments of biology in general and clinical medicine in particular[45].

Zusammenfassung

Zunächst werden primäre und sekundäre Anteile der aktiven Enzymzentren und oxydative Desaminierung definiert. Es wird dann ein allgemeines Verfahren geschildert um durch die Exploration des Substrat- und Inhibitorbereichs das aktive Zentrum der Monoamin-oxydase (MO) zu untersuchen. Phenylalkylamine, Phenylcyclopropylamine und Phenylalkylhydrazine erreichen dann ihre maximale Wirkung, wenn sie aus einem aromatischen Ring und aus einer zweigliedrigen Seitenkette bestehen. Schliesslich wird der Einfluss dieser Studien, die zum Teil ihren Ausgang von der Analyse des Iproniazideffekts nahmen, auf die Erforschung des Stoffwechsels, der Pharmakologie und der Klinik biogener Amine angedeutet.

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CONGRESSUS

Sweden

International Congress on Biophysics

Stockholm, July 31 to August 4, 1961

An International Congress on Biophysics will be held in Stockholm from July 31 to August 4, 1961. The purpose of the meeting is to provide a forum for international communication in the field of biophysics. Participants may include members of national societies of biophysics, medical physics, and related fields, and other scientists interested in pure and applied biophysics. The meeting will be divided between a series of symposia devoted to special topics in biophysics and to presentations of a number of contributed papers in pure and applied biophysics submitted by the participants.

Further information can be obtained from Dr. Bo Lindström at the Department of Medical Physics, Karolinska Institutet, Stockholm 60, Sweden.

Brèves communications - Kurze Mitteilungen
Brevi comunicazioni - Brief Reports

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Electron Impact and Molecular Dissociation Part IV

Considerable interest has beer attached to the behaviour of ether compounds under electron bombardment and a comprehensive survey of the probable modes of fission of alkyl ethers[1] as well as analysis of specific re-arrangements have already been made[2]. More recently BEYNON[3] *et al.*, in the course of a wider survey, have observed the ready elimination of carbon monoxide from diphenyl ether. Little attention has, however, been paid to the possibility of identifying wholly aromatic ethers by their 'cracking-patterns' and this communication is intended to do this.

The aromatic ethers described here include compounds of the type diphenylether, naphthylphenylether, dinaphthylether, and 9-9[1] diphenanthrylether; the full list being included in the Table appended.

These aromatic compounds, in common with the other purely aromatic substances so far investigated, have the parent molecular ions as the most prominent in the spectrum. This is of considerable importance in determining the exact molecular weight of the compound. As already mentioned, there is a substantial fragment ion corresponding to the loss of carbon monoxide. This, in common with the oxygen compounds reported by BEYNON, requires that the elimination shall occur in such a way that the residue, the fragment ion, remains as a unit. Three other classes of aromatic compounds show a similar loss of carbon monoxide, the phenols, quinones, and ketones. The quinones are rather easily detected since the loss of carbon monoxide can and does occur twice over. Thus, for this series, there are abundant ions corresponding to the molecular weight, and the molecular weight less twenty-eight and fifty-six units respectively. The phenols are less easily differentiated from the ethers of corresponding molecular weight as each may eliminate only one carbon monoxide molecule and accordingly there is a parent molecular ion (P) and an abundant P-28 fragment ion. However, an aromatic ether has necessarily two aryl groups unlike a phenol. Accordingly the 'crackingpattern' of an ether will contain a fragment ion corresponding to one or other of the fissions

$$RO^+R^1 \quad \begin{matrix} R^+ + \cdot OR^1 \\ R^{+1} + \cdot OR \end{matrix}$$

or the corresponding fissions with the charge on the other moiety.

Fortunately, with reservations to be detailed below, the production of aryl ions by such fissions is a rather probable process and ether spectra contain prominent fragment ions so derived.

In addition to the fragment ions so far mentioned, there are smaller but still significant ions representing the loss of one or two hydrogen atoms from the parent molecular ion. Such behaviour has previously been noticed in the electron-induced dissociations of aromatic hydrocarbons[4] and have been used as part of the basis for distinguishing among various possible isomers in this series.

Whilst the determination of molecular weight and group thus seems a fairly simple process, the identification of the various possible isomeric aryl radicale in the original ether seems to be a problem of greater difficulty. So far, in the series presently reported, there seems little possibility of confusion amongst the various members. Even this series does, however, allow certain generalisation to be made.

(1) The probability of the loss of carbon monoxide and the appearance of the hydrocarbon residue as a single ion diminishes with the increasing molecular weight of the ether and the increasing complexity of the aryl group.

(2) The fission of the ether ion occurs in the way already described with the production of aryl ions in significant abundance, but with little formation of the corresponding aryloxy ion. In unsymmetrical compounds, this fission greatly favours the formation of the ion of the larger aryl radical as is shown in the fissions of the unsymmetrical phenyl ethers.

(3) In the unsymmetrical ethers two series are available, the three possible naphthyl ethers and *o*- and *p*-phenoxylbiphenyl. In each case, the experimental evidence shows that distinctions amongst these series of isomers may be obtained by a consideration of the fragment ion P-1. The magnitude of this expressed as a percentage of the abundance of the parent molecular ion diminishes in the order $\beta{:}\beta^1$ dinaphthyl $>$ $\alpha{:}\beta$ dinaphthyl $>$ $\alpha{:}\alpha^1$ dinaphthyl and the corresponding function indicates that the order is *p*-phenoxy $>$ *o*-phenoxy in the biphenyl series.

Experimental. The spectra were obtained in the usual way on a Metropolitan-Vickers Ltd. M. S. 2 Mass-Spectrometer employing a repeller potential of 2 K volts and magnetic scanning.

The appropriate ethers were obtained by the following methods. Diphenyl ether was reagent grade, purified by crystallisation. α-naphthyl phenyl, β-naphthyl phenyl, $\alpha{:}\alpha$-dinaphthyl, $\alpha{:}\beta^1$-dinaphthyl[5], $\beta{:}\beta^1$-dinaphthyl[6], 9-phenanthryl phenyl[7] and di-9-phenanthryl[8] ethers were prepared by known methods. The phenoxybiphenyls were prepared by an adaptation of a known method[9].

p-Phenoxybiphenyl. To a solution of 4·5 g sodium methoxide in 90 ml methanol was added 14·2 g *p*-hydroxybiphenyl and 6·0 g diphenyliodonium bromide[10]. After the mixture had been refluxed for 24 h, the ether soluble product was collected and distilled under reduced pressure. The solid distillate, recrystallised from benzene gave 1·37 g (48%) *p*-phenoxybiphenyl, m.pt. 136°C.

[1] F. W. MCLAFFERTY, Anal. Chem. *29*, 99 (1957).
[2] F. W. MCLAFFERTY, Anal. Chem. *31*, 2072 (1959).
[3] J. H. BEYNON, G. R. LESTER, and A. E. WILLIAMS, J. phys. Chem. *63*, 1861 (1960).
[4] R. I. REED, Third Int. Conf. on Coal Science, Falkenberg, April (1959).
[5] F. ULLMAN and P. SPONAGEL, Liebigs Ann. *350*, 90 (1906).
[6] M. E. BERGER, C. R. Acad. Sci., Paris *141*, 1027 (1905).
[7] R. L. HUANG, J. chem. Soc. *1955*, 3295.
[8] F. R. JAPP and A. FINDLAY, J. chem. Soc. *71*, 1115 (1897).
[9] F. M. BERINGER, A. BRIERLEY, M. DREXLER, E. M. GINDLER, and C. C. LUMPKIN, J. Amer. chem. Soc. *75*, 2708 (1953).
[10] F. M. BERINGER, M. DREXLER, E. M. GRINDLER, and C. C. LUMPKIN, J. Amer. chem. Soc. *75*, 2705 (1953).

M/e	$R \cdot O \cdot R^1$									
	9-Phenanthryl 9-Phenanthryl	9-Ph Phenyl	β-Naphthyl β-Naphthyl	β-Naphthyl α-Naphthyl	α-Naphthyl α-Naphthyl	4-biphenyl Phenyl	2-biphenyl Phenyl	β-Naphthyl Phenyl	α-Naphthyl Phenyl	Phenyl *R* Phenyl *R*¹
370	*100*	—	—	—	-	—	—	—	—	—
369	55·1	—	—	—	—	—	—	—	—	—
368	13·7	—	—	—	—	—	—	—	—	—
342	*18·8*	—	—	—	—	—	—	—	—	—
341	16·5	—	—	—	—	—	—	—	—	—
270		*100*	*100*	*100*	*100*	—	—	—	—	—
269	—	—	80·5	77·0	69.4	—	—	—	—	—
268	—	—	24·6	16·9	25·3	—	—	—	—	—
246		—	—	—	—	*100*	*100*	—	—	—
245	-	—	—	—	—	64·5	50·3	—	—	—
244	—	—	—	—	—	16·6	30·7	—	—	—
242	—	*12·9*	*25·2*	*28·8*	*18·6*	—	—	—	—	—
241	—	20·6	22·3	17·8	15·7	—	—	—	—	—
220		—	—	—	—	—	—	*100*	*100*	—
219		—	—	—	—	—	—	61·1	62·5	—
218	—	—	—	—	—	*10·1*	*7·8*	22·3	20·3	—
217	—	—	—	—	—	5·3	8·0	—	—	—
192	-	—		—	—	—	—	*23·0*	*10·3*	-
191	—	—	—	—	—	—	—	30·7	19·5	—
177	8·3	63·8	-	—	—	-	—	—	—	—
170	—	—		—		-	—	—	—	100
169		—	—	—	—	—	—	—	—	73·8
168	—	—	—	—	-	—	—	—	—	37·0
165	18·3	35·1	-	—	—	—	—	—	—	—
153	—	—	—	—		43·8	38·4[a]	—	—	—
151		—	—	—	-	—	—	—	—	—
142	—	—			—	-	-	—	—	*39·3*
141		-	—	—	—	44·4	10·9	—	—	60·7
140	—	-	—	—	—	—	—	—	—	15·5
127			39·6[a]	38·4[a]	28·4[a]	-	—	15·3[a]	11·7[a]	—
126	—	—	—	19·2	14·8	—	—	—	—	—
117		—		—	—	—	—		—	7·0[b]
115	—	-	—	—		12·6	9·5	—	—	17·1
94	—			—	—	—			—	6·8
89	—	-	-	—	—	-	—	—	-	6·0
88	—	—	—		-	—	-	—	—	—
78	—			-		—	—	—	—	—
77	—	—	—	—	—	9·4	10·2[a]	—	—	72·7[a]
76	—	—			—		—	—		—
65		—	-	—	—	—	—	—	-	14·5
63	—	—		—	-	—	—	—	-	8·5
51		-		-	—	—	—	—	—	75·2
50	—	-	—	—	—		-		—	16·2
39		-			—	-	—		-	33·3

Masses underlined *100* represent the abundance of the parent molecular ion. Underlined 25.2 the abundance of the parent minus 28 (P-28) ion. [a] This represents the abundance of one or other of the possible aryl ions from the ether. [b] Represents a metastable ion.

o-Phenoxybiphenyl. The same method was used, but light petroleum b. pt. 60°–80°C was used to recrystallise the ether. This gave 0·91 g *o*-phenoxybiphenyl (35%) m.pt. 37°C.

The author wishes to express his thanks to Dr. R. I. Reed for advice, encouragement, and valuable discussion while this work was being carried out: also to the Institute of Petroleum (Mass Spectrometry Panel) for a maintenance grant.

J. M. Wilson

Chemistry Department, The University, Glasgow, February 15, 1960.

Zusammenfassung

Es wird über die ionischen Hauptbruchstücke in den Massenspektren von zehn Diaryläthern berichtet. Die Intensitäten können zur Identifizierung dieser Verbindungen dienen.

The Structure of Cassamine and Erythrophlamine[1]

The crystalline alkaloids cassamine ($C_{25}H_{39}O_5N$) and erythrophlamine ($C_{25}H_{39}O_6N$), isolated[2] from *Erythrophleum guineense* G. Don, are known[3] to be β-dimethylamino-ethanol esters of two unsaturated acids named respectively cassamic acid ($C_{21}H_{30}O_5$) and erythrophlamic acid ($C_{21}H_{30}O_6$). Cassamic acid contains one double bond $\alpha\beta$ to the carboxyl group, one keto and one methoxyl group. Erythrophlamic acid, in addition to the same functional groups, also carries a hydroxyl group. In both acids the nature of one oxygen atom, the carbon skeleton and the position of the functional groups remained to be determined[4].

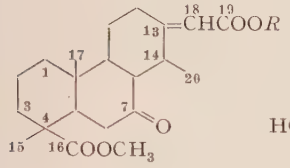
[1] 16th Communication on Erythrophleum Alkaloids. 15th Comm.: B. G. Engel, Helv. chim. Acta *42*, 1127 (1959).

[2] B. G. Engel and R. Tondeur, Exper. *4*, 430 (1948); Helv. chim. Acta *32*, 2364 (1949).

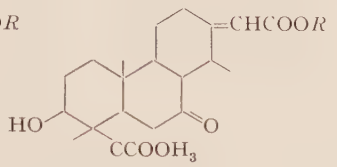
[3] B. G. Engel, R. Tondeur, and L. Ruzicka, Rec. Trav. chim. Pays-Bas *69*, 396 (1950).

[4] The numbering used is that common to steroids and triterpenes.

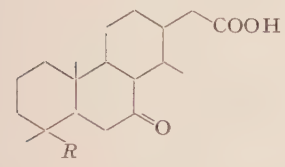
It has now been found that the two acids contain a strongly hindered quaternary carbomethoxy group. Furthermore the following results prove the complete structure of the acids to be Ia for cassamic and IIa for erythrophlamic and consequently Ib and IIb respectively for cassamine and erythrophlamine.



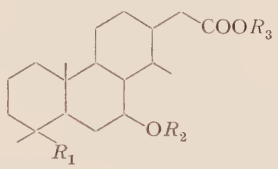
Ia $R = H$
b $R = CH_2CH_2N(CH_3)_2$



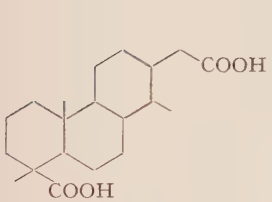
IIa $R = H$
b $R = CH_2CH_2N(CH_3)_2$



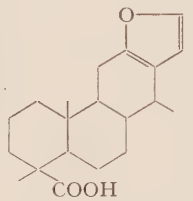
IIIa $R = COOCH_3$
b $R = CH_3$



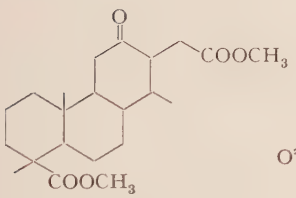
IVa $R_1 = COOCH_3$; $R_2 = R_3 = H$
b $R_1 = COOH$; $R_2 = R_3 = H$
c $R_1 = COOH$; $R_2 = COCH_3$; $R_3 = CH_3$
d $R_1 = COCl$; $R_2 = COCH_3$; $R_3 = CH_3$
e $R_1 = CHO$; $R_2 = COCH_3$; $R_3 = CH_3$
f $R_1 = CH_3$; $R_2 = R_3 = H$



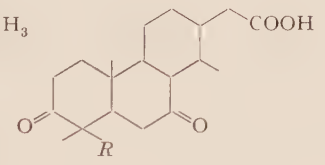
V



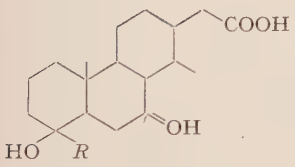
VI



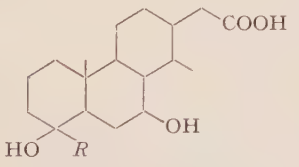
VII



VIIIa $R = CH_3$
b $R = COOCH_3$



IXa $R = COOCH_3$
b $R = CH_3$



Xa $R = COOCH_3$
b $R = COOH$
c $R = CH_3$

1. *Cassamic acid.* a) *The carbomethoxy group.* Hydrogenation of cassamic acid (Ia) over palladium on charcoal yields dihydrocassamic acid (IIIa, $C_{21}H_{32}O_5$, m. p. 152°C, $[\alpha]_D^{22\,5} = +\,50°$C)[6]. Reduction of IIIa with $NaBH_4$ affords the dihydro hydroxy acid IVa ($C_{21}H_{34}O_5$, m. p. 173°C, $[\alpha]_D^{22} = +\,68°$). The latter under energetic alkaline hydrolysis gives rise to the hydroxy dicarboxylic acid IVb ($C_{20}H_{32}O_5$, m. p. 262–263°C, $[\alpha]_D^{21} = +\,46°$ in methanol, pK_{MCS}[7] 7, 18; 8,32). That no rearrangement takes place under the conditions of reaction is shown by the reconversion of IVb into the crystalline starting material IVa by esterification with diazomethane followed by mild hydrolysis.

These experiments establish that cassamic acid contains a quaternary carbomethoxy group.

b) *The carbon skeleton and the position of the quaternary carbomethoxy group.* Dihydrocassamic acid (IIIa) under Wolff-Kishner conditions yields the diacid V, concomitant hydrolysis of the quaternary carbomethoxy group taking place. This dicarboxylic acid V ($C_{20}H_{31}O_4$) m.p. 260–261°C and $[\alpha]_D^{20} = +\,40°$, proved identical with a product prepared from vouacapenic acid (VI)[8,9] by the following route. The keto dimethyl ester VII described by King *et al.*[9], was subjected to thioketal hydrogenolysis followed by vigorous hydrolysis: the same diacid V was obtained.

As the structure of vouacapenic acid has been determined[8,9], the above correlation provides definite proof for the carbon skeleton of dihydro cassamic acid and for the position of the quaternary carbomethoxy group as depicted in formula IIIa. That the acid side chain at C_{13} is unsaturated in cassamic acid itself Ia is shown by the isolation of oxalic acid on ozonolysis.

c) *The position of the carbonyl group.* In another series of reactions the quaternary carboxyl group at C_4 in the hydroxy dicarboxylic acid IVb was converted to a methyl group through the steps: hydroxy dicarboxylic acid IVb → primary monomethylester acetate IVc → acid chloride IVd →[10] aldehydo acetoxy methyl ester IVe → hydroxy acid IVf. The final product of this conversion possessed the empirical formula $C_{20}H_{34}O_3$, m. p. 226°C and $[\alpha]_D^{22} = +\,42°$, and proved to be identical in all respects with an authentic sample of 7-hydroxycassanic acid (IVf). The latter was obtained from 3,7-diketocassanic acid[11] (VIIIa) by Clemmensen reduction followed by treatment of the resulting 7-ketocassanic acid (IIIb) with $NaBH_4$[12].

[5] Optical rotations are in ethanol unless otherwise stated.

[6] Satisfactory microanalyses have been obtained for all compounds mentioned. Spectral properties are in accord with the stated structures.

[7] The notation pK^*_{MCS} denotes the negative logarithm of the apparent dissociation constant in 80% methylcellosolve/water mixture (w/w) as determined by the method of W. Simon, Helv. chim. Acta *41*, 1835 (1958). The determinations were carried out by Dr. W. Simon, ETH, Zurich, whom we wish to thank for his kind co-operation.

[8] F. E. King, D. H. Godson, and T. J. King, J. chem. Soc. *1955*, 1117.

[9] F. E. King, T. J. King, and J. M. Uprichard, J. chem. Soc. *1958*, 3428.

[10] Selective reduction with $LiAl(OBu^t)_3H$, cf. H. C. Brown and R. F. McFarlin, J. Amer. chem. Soc. *80*, 5372 (1958).

[11] L. Ruzicka, G. Dalma, and W. E. Scott, Helv. chim. Acta *24*, 184E (1941).

[12] That only the 3-keto group of 3.7-diketocassanic acid (VIIIa) is reduced follows from the fact that the product of the Clemmensen reduction (IIIb) differs from 3-ketocassanic acid obtained by A. Ronco, Diss. ETH, Zurich (1945) through Wolff-Kishner reduction of dihydrocassaic acid (IXb) and subsequent chromic acid oxidation of the original 3-hydrocassanic acid. The latter is also found to be different from the hydroxy acid IIIb.

This result confirms that cassamic acid has the same carbon skeleton as cassanic acid[9,11,13], proves that the carbonyl group is attached to carbon atom 7[14] and establishes formula IIIa for cassamic acid.

2. *Erythrophlamic acid.* a) *The quaternary carbomethoxy group, the carbon skeleton, and the position of the oxygen functions.* Erythrophlamic acid (IIa) is hydrogenated over a Pd/C-catalyst to the saturated hydroxy keto acid IXa ($C_{21}H_{32}O_6$), m. p. 227–228°C, $[\alpha]_D^{22} = +50°$. By treatment of this saturated hydroxy keto acid with $NaBH_4$, the corresponding dihydroxy acid Xa is obtained ($C_{21}H_{34}O_6$; m.p. 251–252°C, $[\alpha]_D^{22} = +69°$ in methanol). Energetic alkaline hydrolysis of the latter leads to the dihydroxy dicarboxylic acid Xb ($C_{20}H_{32}O_6$; m.p. 283–284°C, $[\alpha]_D^{22} = +37°$ in methanol). The starting material of the energetic alkaline hydrolysis, the dihydroxy acid Xa, is regenerated from the dicarboxylic dihydroxy acid Xb by esterification with diazomethane followed by mild alkaline hydrolysis of the primary carbomethoxy group.

When the dihydroxy dicarboxylic acid Xb is submitted to the same series of reactions as the corresponding compound IVb of the cassamic series (cf. 1c), a dihydroxy acid is obtained ($C_{20}H_{34}O_4$, m.p. 262–265°C, $[\alpha]_D^{21} = 0°$). Comparison with an authentic sample of dihydroxycassanic acid (Xc) from cassaidine[15] showed the two to be identical.

The experiments described above demonstrate the presence of a quaternary carbomethoxy group and determine the positions of the two oxygen atoms in erythrophlamic acid, as well as its carbon skeleton. However, they provide no information on the point of attachment of the quaternary carbomethoxy group, nor do they indicate which of the two hydroxyls of dihydroxycassanic acid (Xc) is present as a keto group in erythrophlamic acid.

b) *The position of the quaternary carbomethoxy group and of the keto and hydroxyl groups.* Dihydroerythrophlamic acid (IXa) is easily oxidized by chromic acid to a diketo acid (VIIIb, $C_{21}H_{30}O_6$, m.p. 203–204°C, $[\alpha]_D^{22} = +13°$). Clemmensen reduction of the latter affords as the main product dihydrocassamic acid (IIIa)[16].

As the keto group in dihydroerythrophlamic acid (IXa) itself is unaffected by Clemmensen reduction, the conversion of the diketo acid (VIIIb) to dihydrocassamic acid (IIIa), together with the preceding one of the dihydroxy acid Xb to dihydroxycassanic acid (Xc) prove structure IIa for erythrophlamic acid itself.

The results of these and related experiments not reported here, as well as their stereochemical implications, will be published in full elsewhere.

We have to thank Dr. T. J. King for a very generous supply of vouacapenic acid and the *Institut National pour l'Etude Agronomique du Congo Belge* (INEAC) in Bruxelles for providing us with the bark of *Erythrophleum guineense* G. Don. The Zurich group acknowledges with thanks the financial help of CIBA AG. in Basle. V.P.A. is indebted to the CIBA *Followship Trust* for a CIBA Fellowship.

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D. W. Mathieson, B. Jaques, G. T. Chapman*, V. P. Arya, and B. G. Engel**

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Zusammenfassung

Es wird gezeigt, dass Cassamin und Erythrophlamin, zwei kristalline Nebenalkaloide aus *Erythrophleum guineense* G. Don, neben den anderen schon bekannten Sauerstoffunktionen je eine stark gehinderte Carbomethoxy-Gruppe enthalten. Durch Überführung in Verbindungen bekannter Konstitution wird Formel Ib für Cassamin und Formel IIb für Erythrophlamin bewiesen.

[13] L. G. Humber and W. I. Taylor, J. chem. Soc. *1955*, 1044.

[14] For the position of the oxygen atoms in the cassaine series cf. W. J. Gensler and G. M. Sherman, Chem. & Ind. *1959*, 223; J. Amer. chem. Soc. *81*, 5217 (1959). – R. B. Turner, E. G. Herzog, R. B. Morin, and A. Riebel, Tetrahedron Letters No. 2, 7 (1959). – V. P. Arya and David W. Mathieson, J. chem. Soc. *1959*, 3623.

[15] L. Ruzicka and G. Dalma, Helv. chim. Acta *23*, 753 (1940).

[16] Cf. the analogous reduction of 3.7-diketocassanic acid to 7-ketocassanic mentioned under 1c.

Inhibitory Action of some Compounds on Staphylococcal Coagulase

The present communication describes data concerning the action of several substances on the *in vitro* activity of staphylococcal free coagulase. Among these were: 1[1] antibiotics, 6 chemotherapeutics, 3 antiseptics, ingredients of the preserving solution used in the blood banks, 2 nucleic acids and their salts, 5 dextrans and 29 inorganic salts. All substances were crystalline or the best purity available.

Determination of the coagulase titer, coagulase standard, and selection of plasma were the same as previously described[1,2]. Strain J-373 (*Staphylococcus aureus*, strongly coagulase-positive) was grown in Roux bottles containing 100 ml of brain heart infusion broth with the addition of 'ion mixture'[3]. After 18 h incubation, the culture was divided into two parts: one was centrifuged at 5000 r.p.m. for 30 min and the supernatant was used as the source of coagulase; the second part was shaken and added with living cells as the second source of coagulase. Two series of tubes were prepared. Each tube contained 0·5 ml of saline in which dilution of the substance studied were made. Concentrations varied from 0·46 to 1000 γ/ml. To the first series of tubes 0·5 ml of supernatant, and to the second – of living culture, were added. The tubes were shaken and placed in the incubator (37°C) for 1 h. After this time 0·5 ml of citrated rabbit plasma was added to each tube. They were shaken and incubated at 37°C. Readings were made after 2, 6, and 24 h. All procedures were performed in sterile conditions. The third set consisted of the same amount of tubes containing 0·5 ml of saline, 0·5 ml of supernatant, and 0·5 ml of rabbit plasma and served as control showing the actual coagulase titer.

The substances possessing the inhibitory action on staphylococcal free coagulase are listed in the Table.

Antibiotics had a very small effect on coagulase activity in few instances only. Out of 19 antibiotics studied, spiramycin and oleandomycin only in the highest concentration used (1000 γ/ml) – inhibited the clotting. Bacitracin and cycloserine slightly delayed the appearance of the clot. Other antibiotics were completely without effect and clots were obtained promptly, which were as solid as in the control. Isoniazid, and sulfonamides did not also exert a pronounced action on coagulase activity. The exceptions were:

[1] J. Jeljaszewicz, Acta microbiol. Polon. *7*, 17 (1958).

[2] J. Jeljaszewicz, Med. Dośw. Mikrobiol. *10*, 287 (1958).

[3] M. Tager and H. Hales, Yale J. biol. Med. *20*, 41 (1947).

[4] Details about the preserving solution were obtained through the courtesy of Mr. J. Meczyński from the Poznań Blood Transfusion Station.

[5] K. Wlodarczak and J. Jeljaszewicz, Arch. Immun. Ter. Dośw., in press (1960).

Substances disturbing the *in vitro* coagulase activity

Substance	Minimal concentration causing coagulase inhibition in γ/ml
Sodium ethylmercurithiosalicylate	0·46
Albucid	0·46
Conserving solution	0·46 ml
Nucleic acid	0·46
Desoxyribonucleic acid	0·46
$CuSO_4$	1·85–7·82[a]
Na_3N	7·82–31·25[a]
$K_2Cr_2O_7$	31·25–125·0[a]
$ZnSO_4$	125·0
$CdSO_4$	215·0– 250·0[a]
$MgCl_2$	125·0–1000·0[a]
Phenol	125·0–1000·0[a]
$H_3P/Mo_3O_{10}/_4$	125·0–1000·0[a]
$PtCl_3$	500·0
$BaCl_2$	500·0
$HgCl_2$	500·0
KJ	500·0
$Al_2/SO_4/_3$	500·0
Sulfamethoxypirydazine	500·0
NH_4Cl	500·0–1000·0[a]
$K_4/Fe/CN/_6$	1000·0
Sulfanilamide	1000·0
Oleandomycin	1000·0
Spiramycin	1000·0

[a] In some cases the degrees of inhibition of coagulase in supernatant and living culture–differed.

sulfanilamide, which acted in the highest concentration and sulfamethoxypiradazine, which inhibited clotting also in a rather great quantity (500 γ/ml). The most marked action among antiseptics was that of sodium ethylmercurithiosalicylate ('thiomersalate', 'merthiolate'). This compound even in the lowest quantity (0·46 γ/ml) completely prevented clotting. The action of phenol and rivanol was slightly inhibitory.

The preserving solution used at the blood banks for transfusion purposes (it is mixed in proportions 1:5 with blood and consists of 30 g of sodium citrate, 30 g of glucose, 5 g albucid, 0·03 g rivanol, 22 ml of 50% HCl, and distilled water to 1000 ml, pH = 4·9–5·1)[4] was strongly inhibitory to coagulase. Even 0·46 ml of it prevented the clotting. It is rather interesting because all tests connected with physiological blood clotting were correct.

Nucleic acids also completely inhibited the coagulase action. Dextrans, with the exception of two samples which slightly delayed the clotting process, did not inhibit the coagulase.

Out of 29 inorganic salts, only 17 influenced coagulase at rather higher concentrations [NH_4Cl, $ZnSO_4$, $CdSO_4$, $Al_2(SO_4)_3$, $CuSo_4$, $K_4Fe(CN)_6$, KJ, $HgCl_2$, Na_3N, $BaCl_2$, $MgCl_2$, $K_2Cr_2O_7$, $FeCl_3$, $PtCl_3$, and $H_3P(Mo_3O_{10})_4$]. Some differences between the inhibition of coagulase concerned in the supernatant and living culture were observed in the experiments with salts.

The mechanism of these inhibitions is unknown in relation to the action of coagulase and physiological blood clotting.

Detailed results and a critical review on the problem described will be published later[5].

J. Jeljaszewicz and K. Wlodarczak

Department of Microbiology, Medical Academy, Poznań and Regional Sanitary and Epidemiological Station, Poznań (Poland), February 4, 1960.

Zusammenfassung

Es wurde der Einfluss von 67 Substanzen (Antibiotika, Chemotherapeutika, Sulfonamide, Antiseptika, Dextrane, Nukleinsäuren und Metallsalze) auf die Aktivität der freien Koagulase der Staphylokokken untersucht, worunter 24 als Inhibitoren auftraten.

Glutamic Acid-Alanine Transaminase in Tobacco Leaves Infected with TMV

In one of the former works, active glutamic acid-alanine transaminase and decarboxylase of glutamic acid in leaves of tobacco and other plants was proved[1].

Later on it was found that decarboxylase, in plants inoculated with TMV, increases in intensity[2].

Here the preliminary results are shown dealing with investigation in transamination in tobacco inoculated with tobacco mosaic virus (TMV). The method of investigation was similar to that described in the former account, and therefore only the most important finding are given here.

A sample (2 g) of leaves, picked in the morning, was ground in a mortar together with 5 ml $M/15$ phosphate buffer pH 7·6. The homogenate thus prepared in a quantity of 3 ml was mixed in a test tube with 2 ml glutamic acid (concentration 0·02 M) and 2 ml of pyruvic acid of the same concentration. The sample was divided into two parts; one of which was immediately boiled and the other with a delay of 45 min after incubation at 27°C. After boiling, they were filtered and the product was pipetted onto Whatman No. 1 filter paper and chromatographed in 80% phenol.

The activity of transamination was estimated by the increase of alanine. The chromatograms were coloured with ninhydrin (0·5) and the coloured areas which developed were eluted; measurements were made using Pulfrich photometer according to Kretovich[3].

The tested plants was *Nicotiana tabacum* v. White Burley, grown in a glass-house. It was inoculated with TMV and with the use of carborundum, according to the generally accepted rule.

The results are shown in the diagram. It is evident that, on the second day after inoculation, the transamination increases greatly. On the third day, it drops more than 50% in comparison with sound leaves. The activity of glutamic-alanine transaminase remains on that level until the mosaic appears on the leaves. Later on (4 weeks after inoculation) it increases to 75%.

Nowadays, the generally accepted conviction is that transamination is the cardinal process in the synthesis of amino acids and the inhibition of it may be incomprehensible in the case of plants synthetising TMV.

After the inoculation of tobacco with TMV, its leaves become yellowish day by day. It may be the evidence of decreased activity of some metabolic processes. On the other hand, however, intensive virus synthesis and increased activity of some enzymes[4] and processes are to be observed[5].

It is very likely that inhibition of transamination is due to a peculiar change of plant metabolism caused by the synthesis of extraneous proteins.

[1] M. Gubański, Acta Soc. bot. Poloniae *27*, 291 (1958).

[2] M. Gubański, Nature *186*, 657 (1960).

[3] W. L. Kretovich and J. V. Uspenskaya, Biochem. (USSR) *23*, 248 (1958).

[4] C. Martin, C. R. Acad. Sci., Paris *246*, 2026 (1958).

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Investigations to elucidate the process and its connection with virus synthesis are going on.

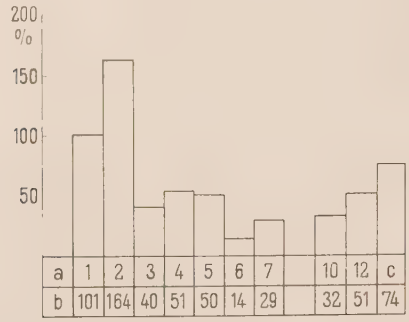

Fig. 1. Activity of transaminase on dry weight

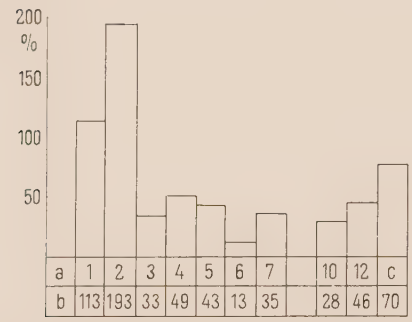

Fig. 2. Activity of transaminase on fresh weight

a) Days after infection. b) Percentage of transamination in inoculated leaves in terms of the activity of sound plants (100%). c) 4 weeks after infection.

We are very grateful to Prof. Dr. W. Moycho for his assistance and critical suggestions.

M. Gubański and R. Kurstak

Department of Plant Physiology, University of Lódź (Poland), January 25, 1960.

Zusammenfassung

In den mit Mosaikvirus infizierten Tabakblättern wurden im Vergleich mit den gesunden bedeutende Veränderungen in der Glutamin-alanin-transaminase-Aktivität festgestellt. Zwei Tage nach Infektion erfolgt zunächst Zunahme, dann Abnahme der Enzymaktivität.

10. On the Sulphurylation of Mono-, Di-, and Trihydric Phenols

The sulphurylation of a large number of phenols and phenol derivatives has been studied by earlier workers. This has been done in *in vivo* experiments on various species[1], or *in vitro* in slices of various tissues[2,3], as well in particle-free media containing sulphate-activating and sulphate-transferring enzymes[4–7].

The last-mentioned technique was used in the present comparative study on the sulphurylation of a number of mono-, di-, and trihydric phenols. S^{35}-labelled sulphate was used as tracer. The formation of conjugated sulphates in the incubating medium was followed by means of two-dimensional paper chromatography and electrophoresis,

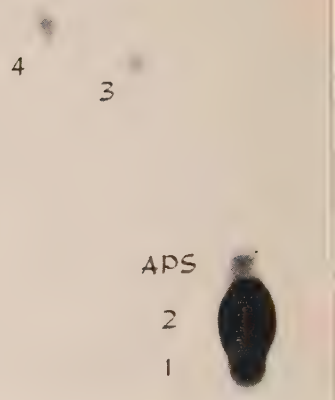

Fig. 1. Basic chromatographic pattern of the incubating medium. Spots 1 and 2 correspond to inorganic sulphate and active sulphate (PAPS) and spots 3 and 4 to unknowns derived from the liver extract.

both techniques combined with autoradiography on Gevaert X-ray film. Detailed descriptions of the methods used have been given elsewhere[8].

The following types of experiment were run:

(a) Incubation for 2 h at 37°C in the complete system (particle-free rat liver supernatant buffer[9], ATP, Mg ions and S^{35}-labelled sulphate) of various concentrations (0·1 to 2·0 *mM*) of each of the following phenols: phenol, catechol, resorcinol, hydroquinone, pyrogallol, and phloroglucin.

(b) Incubation as above of the complete system, with the phenol solutions replaced by water (control).

(c) Incubation as above of the complete system, with the S^{35}-sulphate solution replaced by water, and the phenol solutions replaced by S^{35}-labelled monosulphates of the aforementioned phenols obtained by biochemical synthesis.

(d) Acid hydrolysis in 0·1 *N* HCl for 0–2 h of eluted S^{35}-labelled spots obtained in the experiments listed above. The hydrolysates were submitted to paper chromatography and paper electrophoresis combined with autoradiography.

The results of these experiments are illustrated in Figures 1–3. The basic chromatographic pattern of the incubating medium in the control experiments (b), shown in Figure 1, was essentially the same as described earlier[8]. In addition to the inorganic + PAPS spot and the APS spot in the right lower corner, a few additional spots due to the sulphurylation of sulphate acceptors present in the rat liver supernatant are visualized. In the presence of

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[9] A. B. Roy, Biochem. J. *63*, 249 (1956).

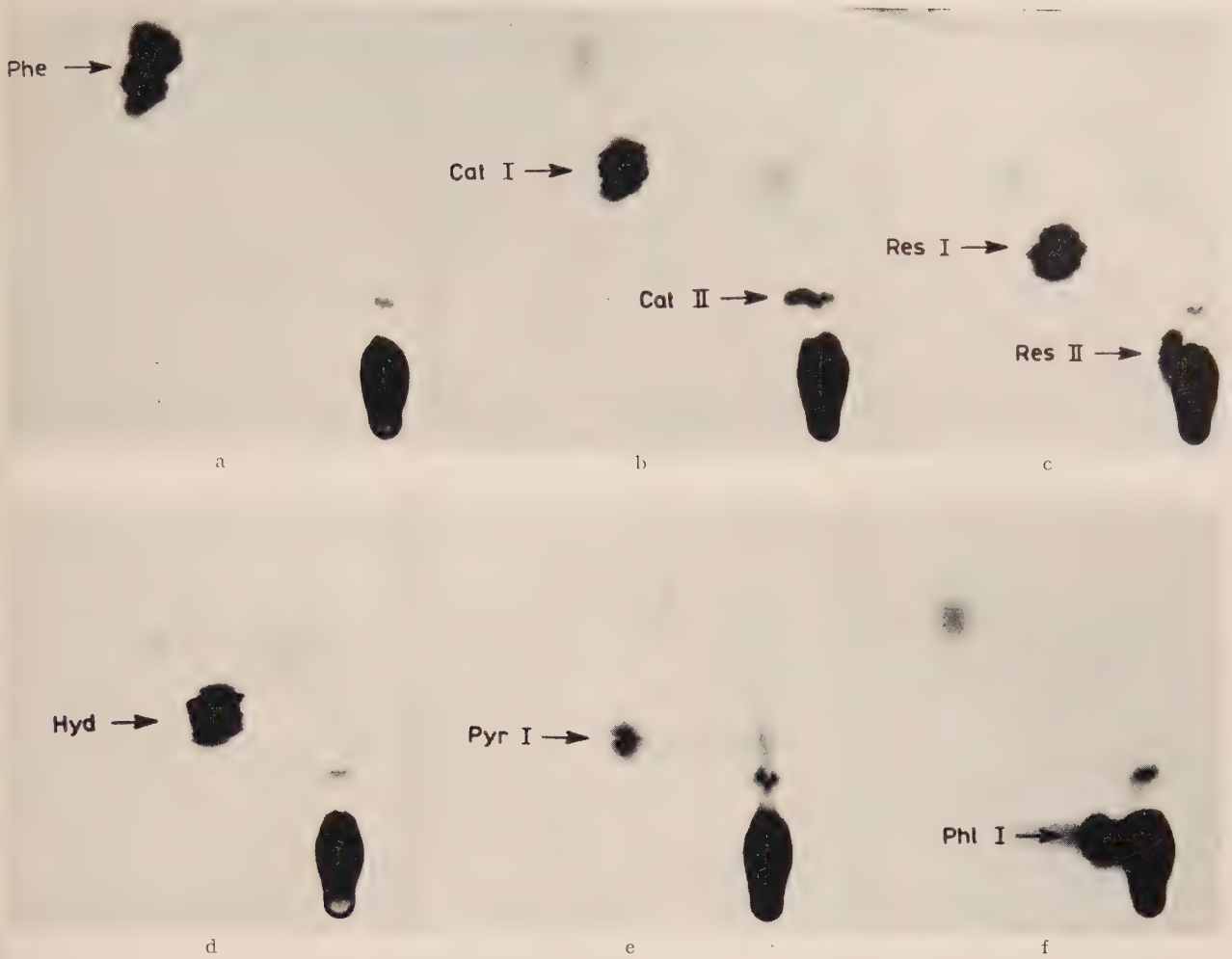

Fig. 2 Chromatographic pattern of the incubating medium in the presence of phenol (Phe), catechol (Cat), resorcinol (Res), hydroquinone (Hyd), pyrogallol (Pyr), and phloroglucin (Phl). Phenol-induced spots indicated by arrows.

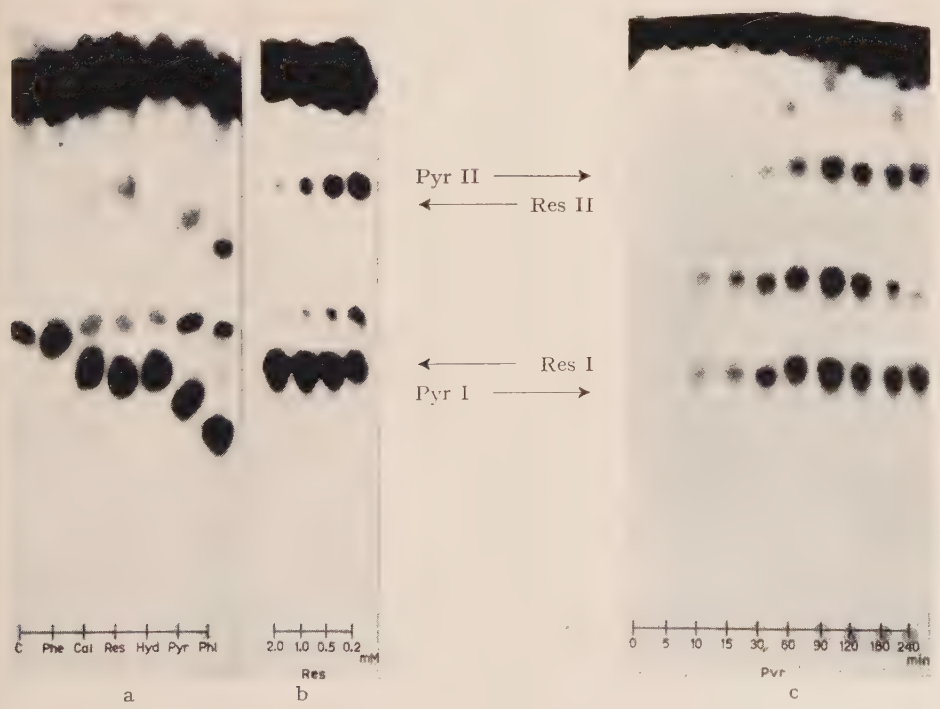

Fig. 3. a) Electrophoretic pattern of the incubating medium from a control experiment (C) and in the presence of various phenols (abbreviations as in Fig. 2). b) Patterns obtained with various amounts of resorcinol present in the medium (0·1–2 *mM*). c) Patterns obtained with pyrogallol (1·0 *mM*) in the medium during incubation for various lengths of time (0–2 h).

each of the phenols (a), one or (in the case of the dihydric phenols catechol and resorcinol) two new main spots appeared on the chromatograms (Fig. 2). Fig. 3A shows the electrophoretic pattern of the same series of experiments. When compared to the control (on the left), all phenols tested induced the formation of one new spot (Phe I to Phl I) in the central region of the electrophoretic strip. In addition, in the experiments on each of the dihydric phenols catechol and resorcinol and the trihydric pyrogallol and phloroglucin, a second fast-moving spot appeared (Cat II, Res II, Pyr II and Phl II). After elution of the spots denoted as Cat II and Res II on the chromatograms, these spots were found to migrate to the Cat II and Res II regions, respectively, on the electropherograms. When the eluted spot in the right lower corner of the chromatograms from the pyrogallol and phloroglucin experiments was submitted to electrophoresis, new spots (in addition to the expected PAPS and inorganic sulphate spots) were observed in the Pyr II and Phl II regions, respectively.

Figure 3B shows the formation of Cat I and II, respectively, during incubation for 2 h in the presence of various amounts of catechol in the medium (0·1–2 *mM*). At a low concentration of catechol, the S^{35} incorporation in Cat II was higher than in Cat I, whereas a high concentration produced the reverse picture.

Figure 3C illustrates the formation of Pyr I and Pyr II, respectively, during incubation of 1·0 *mM* of pyrogallol for various lengths of time (0–2 h). In both spots, sulphate incorporation increased as a function of time.

In the series of experiments (c) where the S^{35}-sulphate and the phenol solutions in the incubating media were replaced by the S^{35}-labelled compounds of Phe-Phl I considered to constitute the monosulphates of respective phenols, no indication was obtained of a formation of the aforementioned fast-moving spots (Cat II, Res II, Pyr II, Phl II).

Acid hydrolysis under various conditions of all the S^{35}-labelled spots listed above, followed by electrophoretic or chromatographic investigation of the split products, resulted in appearance of no labelled compounds, but of inorganic sulphate or of the inorganic + the unhydrolyzed compounds tested. In the case of Res II and Pyr II, a formation of Res I and Pyr I from these compounds was produced by weak acid hydrolysis.

Incubation of a series of mono-, di-, and trihydric phenols in a sulphurylating system thus resulted in formation of a number of sulphate conjugates. Each of the phenols tested induced formation of a sulphate conjugate which migrated in electrophoresis, under the conditions used, to a characteristic position in the central part of the electrophoretic strip. These compounds, which are easily hydrolyzed in hydrochloric acid, giving inorganic sulphate, are considered to represent monosulphates of the corresponding phenols. From two of the three dihydric phenols (catechol and resorcinol) and both the trihydric phenols tested (pyrogallol and phloroglucin), an additional, characteristic, fast-moving electrophoretic spot was obtained which, judging by its electrophoretic mobility, seemed to contain two negative charges. It seems reasonable to believe that these compounds, which also give inorganic sulphate in acid hydrolysates, represent disulphates of the respective phenols. Some support to this view was given by the fact that a formation of monosulphates (Res I and Pyr I) + inorganic sulphate could be demonstrated when eluted specimens of these fast-moving compounds (Res II and Pyr II) were subjected to weak acid hydrolysis. On the other hand, attempts to synthetize these compounds by sulphurylation of the corresponding S^{35}-labelled monosulphates have so far been unsuccessful.

This study was aided by research grants from the Swedish Medical Research Council and the National Institutes of Health, Bethesda, Md., U.S.A. (A-2568 C1).

A. Vestermark and H. Boström

The Wenner-Gren Institute, Department of Metabolic Research, University of Stockholm (Sweden), December 9, 1959.

Zusammenfassung

Die Bildung der Monosulfate bzw. Disulfate in einer Reihe von Phenolen ist auf Grund der *in-vitro*-Versuche mit einem partikelfreien sulfurylierenden Enzymsystem diskutiert worden. S^{35}-markiertes Sulfat wurde als Tracer-Substanz gebraucht. Die gebildeten Sulfatester sind mit zweidimensionaler Papierchromatographie und papierelektrophoretisch getrennt und mittels Autoradiographie lokalisiert worden.

Tyrosine Phosphate on Paper Chromatograms of *Drosophila melanogaster*

Several years ago Hadorn and Mitchell[1] presented results of a study, by paper chromatography, of the ninhydrinreacting components of *Drosophila melanogaster*. In this work and in a number of subsequent investigations[2–5] several substances not corresponding to positions of known amino acids were observed. These were tentatively concluded to be peptides since they gave more than one amino acid on hydrolysis, but mixtures of amino acid derivatives were also possible. Recently, during an examination of this problem, Mitchell and Simmons (unpublished) isolated from *Drosophila* larvae a substance identified as tyrosine-O-phosphate and since this is a major ninhydrin-reacting component in *Drosophila*, its position in the standard chromatographic system is of some interest.

For comparisons, synthetic tyrosine phosphate was prepared according to the method of Pasternak and Grafl[6] and the natural compound was obtained by fractionation of a larval extract on a Dowex 50 column (Mitchell and Simmons, unpublished). These two products had identical absorption spectra in UV light (in 0.1 *M* HCl: max., 265 mμ; inflection, 210 mμ; min., 240 mμ) and behaved as a single substance on co-chromatography in *n*-propanol: 1% ammonia (2:1; $R_f = 0.14$). In order to determine which component from a *Drosophila* extract corresponds to tyrosine phosphate in the two dimensional chromatographic system that has been used extensively in this laboratory[2–5], an extract was prepared and chromatographed alone and in combination with each the synthetic and the natural substance. The tyrosine phosphate moved in each case to the position that had been designated as peptide 1 plus peptide 2[2–5]. As a further check of identity, material from this position on a chromatogram of larval extract

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[8] P. S. Chen, J. Insect. Physiol. *2*, 38 (1958).
[9] National Science Foundation Senior Fellow-Biology Division, California Institute of Technology, Pasadena, California.

alone was eluted with water and subjected to paper electrophoresis (Veronal-Acetate buffer, pH 8.6; 200 V, 3.8 ma) The major portion of the ninhydrin-reacting material (more than 80%) migrated identically with the synthetic and natural tyrosine phosphate preparations (5.0 cm in 3 h).

These results show clearly that the component from *Drosophila* that has been designated $P_1 + P_2$[2–5] consists mainly of tyrosine-O-phosphate.

Ninhydrin-positive components of similar R_f-values have also been found on chromatograms of *Ephestia*- and *Culex*-larvae[7,8]. Studies are now in progress to see if tyrosine-O-phosphate is really present in these insects.

H. K. Mitchell[9], P. S. Chen, and E. Hadorn

Zoologisches Institut der Universität Zürich (Switzerland), July 7, 1960.

Zusammenfassung

Es wird gezeigt, dass die auf zweidimensionalen Chromatogrammen von *Drosophila melanogaster* auftretenden Ninhydrin-positiven Flecken, die als $P_1 + P_2$ (Peptide) bezeichnet wurden, sehr viel freies Tyrosin-O-phosphat enthalten.

The Esterase Activity of Dog's Colostrum

Milk and colostrum from most mammals have low esterase activity. In a few instances, however, milk was found to exhibit high esterase activity, i.e., the colostrum of dog[1] and cow[2], and the colostrum and milk of swine[3–5]. Evidence was presented that the enzyme responsible for this activity is cholinesterase, which is lacking in these secretions of other mammals studied[6,1,7]. No biological explanation is known so far for this species difference in biochemical behaviour, and the question is still open whether esterase-active colostrum contains other esterases in addition to cholinesterase.

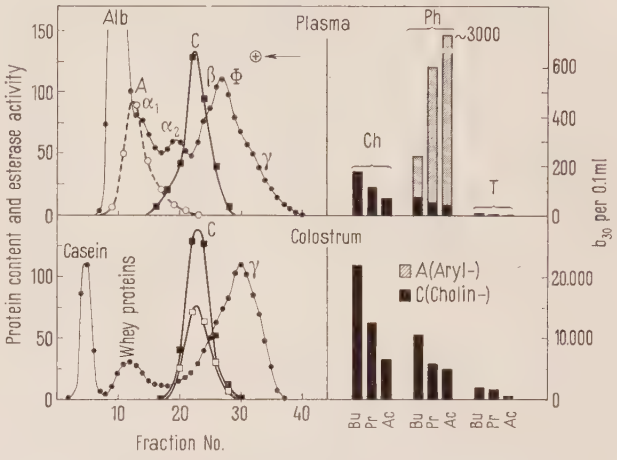
This report presents evidence that the cholinesterases present in bitch's blood plasma and colostrum are identical, and that cholinesterase is the only esterase present in colostrum of this species.

Colostrum was obtained by manual expression very soon after delivery, and its esterase activity measured by the Warburg technique using the acetyl, propionyl, and butyryl esters of choline, phenol, and glycerol (triglycerides). Preparative electrophoretic separation was performed in cellulose columns[8]. For comparison, blood plasma from the same bitch was analysed similarly.

Colostrum was 100 times more active in hydrolysing choline esters than was blood plasma from the same bitch (Fig.). In both cases, butyrylcholine was hydrolysed at the highest rate The triglycerides behaved similarly, but the hydrolysis rates were approximately 12 times lower for colostrum and plasma. As was previously demonstrated for dog plasma[8], cholinesterase was alone responsible for the hydrolysis of tributyrin. The activity of bitch's colostrum in the hydrolysis of tributyrin was completely abolished by 10^{-5} *M* physostigmine, suggesting that cholinesterase of this secretion is the only esterase present which hydrolyses tributyrin.

Phenyl acetate was hydrolysed at about the same rate by colostrum and plasma, but phenyl butyrate was split at a 50 times higher rate by colostrum than by plasma. These differences in specificity of the two esterase sources towards phenyl esters were explained when active proteins were electrophoretically separated (Fig.). Dog plasma contained, in addition to a butyrylcholinesterase, an acetylarylesterase which was absent in colostrum. Evidence was obtained by using seperated enzyme fractions and selective esterase inhibitors that the hydrolysis of phenyl esters by dog plasma was due partly to butyrylcholinesterase. Colostrum gave on electrophoresis only one esterase peak which was due to butyrylcholinesterase, and had the same electrophoretic mobility as the plasma cholinesterase. This enzyme was alone responsible for the hydrolysis of all esters studied as substrates with colostrum.

The butyrylcholinesterase of bitch's colostrum probably originates from blood plasma. Prenatal colostrum, obtained one of the last days of pregnancy, had the same, or slightly lower, cholinesterase activity as postnatal colostrum. In constrast to plasma arylesterase, cholinesterase can pass unchanged into the secretion of bitch's mammary glands. A similar observation was reported previously for sow's colostrum[5], which contains a butyrylcholinesterase in high concentration, but no arylesterase. In contrast to sow's milk, which has the same activity as colostrum during the whole lactation period, the activity of bitch's milk is several hundred times lower than



Electropherograms and specificity of esterases of bitch's colostrum and blood plasma. Distribution of protein and esterase activity (to the left) after electrophoresis of 2.0 ml of diluted (1:4) fat-free colostrum and 1.0 ml of plasma, performed over a period of 16 h in a 1.5 cm × 40 cm cellulose column in veronal buffer (pH 8.4, *I* 0.1) at 10°C with an applied voltage of 300 V. Displacement from the column in 1.5 ml fractions. Thin line, ●—●: relative protein contents (optical density of the Folin colour). Heavy lines: esterase activity, b_{30}; ○---○, phenyl acetate (0.015 ml fractions of plasma); ■—■, butyrylcholine (0.6 ml, plasma; 0.01 ml, colostrum); □—□, tributyrin (0.1 ml, colostrum). In the case of colostrum, one peak only was obtained with phenyl butyrate (0.02 ml fractions) and this peak was identical with that obtained with butyrylcholine. Esterase activities of original material (to the right) are expressed in b_{30} values per 0.1 ml and were obtained with the butyrate (Bu), propionate (Pr), and acetate (Ac) of choline (Ch), phenol (Ph) and glycerol (T, triglycerides).

[1] R. A. McCance, A. O. Hutchinson, R. F. A. Dean, and R. E. H. Jones, Biochem. J. *45*, 493 (1949).

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[7] A. Alm and K.-B. Augustinsson, Acta physiol. scand. *39*, 203 (1957).

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bitch's colostrum. It was also demonstrated that during the first days of suckling, the plasma cholinesterase of puppies increases[1], but this is not the case for the plasma cholinesterase of piglets[5]. This species difference, as well as the functional role of colostrum (and milk) cholinesterase, are still unsolved problems.

K.-B. Augustinsson

Institute of Organic Chemistry and Biochemistry, University of Stockholm (Sweden), April 21, 1960.

Zusammenfassung

Es wird gezeigt, dass die Cholinesterasen im Blutplasma und Colostrum von Hündinnen identisch sind, und dass Cholinesterase die einzige im Colostrum vorhandene Esterase dieser Spezies ist.

Rifomycin IX[1]
Two New Antibiotics of Rifomycin Family: Rifomycin S and Rifomycin SV

Preliminary Report

It has been previously reported[2,3] that the antibacterial activity of aqueous solutions of rifomycin B increases with aging when the samples are exposed to air at room temperature. This property of rifomycin B is shown also by rifomycin O, a product which can be obtained by oxidation of rifomycin B and can be reduced again into rifomycin B[4].

The products found in activated solutions of both rifomycin B and rifomycin O appear to be the same substance[4].

Preliminary data are reported here concerning some properties of this activation product which was named by us rifomycin S. Rifomycin S cristallizes from methanol as yellow-orange crystals, m. p. 179–181°C (dec.). $[\alpha]_D^{20} = +476°C$ ($c = 0.1$, methanol). Rifomycin S has a weakly acidic nature (pH 1/2 = 7.2; equivalent weight 685); its sodium salt is violet. Rifomycin S in buffer phosphate solution pH 7.3 shows absorption maxima at 317 mμ ($E_{1\,cm}^{1\%} = 426$) and at 525 mμ ($E_{1\,cm}^{1\%} = 62$).

Analysis: C 63.34; H 6.79; N 2.18; O 27.80; OCH_3 4.50; $COCH_3$ 6.10. Calc. for $C_{37}H_{47}NO_{12}$ (m.w. 697.75) [proposed]: C 63.68; H 6.79; N 2.01; O 27.51; 1 OCH_3 4.45; 1 $COCH_3$ 6.16.

Rifomycin S can be reduced by ascorbic acid into another antimicrobial substance, rifomycin SV. Rifomycin SV is a yellow-orange crystalline substance, decomposes at 140°C and does not melt until 300°C. $[\alpha]_D^{20} = -4°$ ($c = 1.0$, methanol). Rifomycin SV has an acidic nature (pH 1/2 = 2.7; equivalent weight 685); its sodium salt is orange-red. Rifomycin SV in buffer phosphate solution pH 7.3 shows absorption maxima at 223 mμ ($E_{1\,cm}^{1\%} = 565$), 314 mμ ($E_{1\,cm}^{1\%} = 309$) and 445 mμ ($E_{1\,cm}^{1\%} = 188$).

Rifomycin SV can be transformed again, by oxidation, into rifomycin S. Analysis: C 62.85; H 7.15; N 2.08; O 28.01; OCH_3 4.40; $COCH_3$ 6.15. Calc. for $C_{37}H_{49}NO_{12}$ (m. w. 699.77) [proposed]: C 63.50; H 7.06; N 2.00; O 27.44; 1 OCH_3 4.43; 1 $COCH_3$ 6.15.

Both rifomycin S and rifomycin SV show high activity against Gram-positive bacteria and mycobacteria. In the Table I, the minimal inhibitory concentrations of the two antibiotics against a limited number of strains is reported.

While rifomycin S and rifomycin SV show practically identical antibacterial spectra, they have different acute toxicities. The LD_{50} values in mice by different administration routes are reported in Table II. Mice infected with lethal doses of virulent strains of *Streptococcus haemolyticus, Diplococcus pneumoniae,* or *Staphylococcus aureus* are protected by subcutaneous or oral administration of rifomycin SV.

Tab. I. Antibacterial activity of rifomycin S and rifomycin SV

Microorganism	Minimal inhibitory concentration γ/ml	
	Rifomycin S	Rifomycin SV
Micrococcus aureus	0.005	0.005
Streptococcus faecalis	0.09	0.05
Streptococcus haemolyticus	0.0025	0.0025
Bacillus subtilis	0.075	0.075
Proteus vulgaris	25	25
Escherichia coli	12	25
Pseudomonas aeruginosa	25	50
Klebsiella pneumoniae	25	25
Mycobacterium tuberculosis H 37 Rv	0.05	0.05
Candida albicans	> 100	> 100

Tab. II. Acute toxicity of rifomycin S and rifomycin SV in mice

	Administration route	LD_{50} (mg/kg) values and confidence limits ($p = 0.05$)
Rifomycin S	i. v.	122 (108–138)
	i. p.	258 (243–273)
	os	>3000
Rifomycin SV	i. v.	550 (482–627)
	i. p.	625 (579–675)
	os	2120 (1876–2395)

P. Sensi, M. T. Timbal, and G. Maffii

Research Laboratories, Lepetit S. p. A., Milano (Italy), April 8, 1960.

Riassunto

Da soluzioni di rifomicina B e rifomicina O, che presentano il caratteristico comportamento di un incremento del titolo microbiologico con il tempo, è stato isolato un nuovo antibiotico chiamato rifomicina S. La rifomicina S, per blanda riduzione con acido ascorbico, viene trasformata in rifomicina SV.

[1] P. Sensi, C. Coronelli, and A. Binaghi, Rifomycin. VIII, Farmaco Ed. Prat. (Pavia) *15*, 292 (1960).

[2] P. Sensi, A. M. Greco, and R. Ballotta, Rifomycin. I, Antibiotics Annual 1959-1960 (Medical Encyclopedia, Inc., New York 1960), p. 262.

[3] M. T. Timbal, Rifomycin. II, Antibiotics Annual 1959–1960 (Medical Encyclopedia Inc., New York 1960), p. 271.

[4] P. Sensi, R. Ballotta, and A. M. Greco, Rifomycin. V, Farmaco Ed. Sci. (Pavia) *15*, 228 (1960).

Self-Fertilisation in *Ophryotrocha labronica*

Ophryotrocha labronica (Bacci and La Greca[1]) is a newly described species which has a haploid chromosome complement of three metacentric chromosomes[2]. It is protandrous hermaphroditic and it has been discovered in the course of new researches on sex determination of *Ophryotrocha*[3].

The first appearence of oocytes in this species takes place at a medium length of 13 chaetigerous segments, which thus differs considerably from the means observed in the geographic subspecies of *Ophryotrocha puerilis*, namely *O. puerilis puerilis* of the Mediterranean and *O. puerilis siberti* from the Atlantic. In the former, oocytes first appear at a mean length of 18,21 segments, in the latter at 20,36 chaetigerous segments[4].

Cytological research has demonstrated the presence of male pronuclei in oocytes which were freshly laid by isolated individuals (Fig. 1), and this observation rules out the possibility of parthenogenetic development in such instances. The anaphase of the first maturation was still progressing in the inseminated eggs.

A study of the sequence of sex phases explains the origin of self-fertilisation in *O. labronica*: young individuals of 7–8 chaetigerous segments sometimes show an intense primary spermatogenesis. Spermatogonia and spermatocytes adhere to the dissepiments and to the intestinal walls, and sperms and spermatids fill out the coelome cavities. A few nutrient cells are present in the gonads and oocytes begin to grow in individuals of 9–10 segments. Germinal male elements are gradually eliminated and they are totally absent when oocytes attain medium sizes.

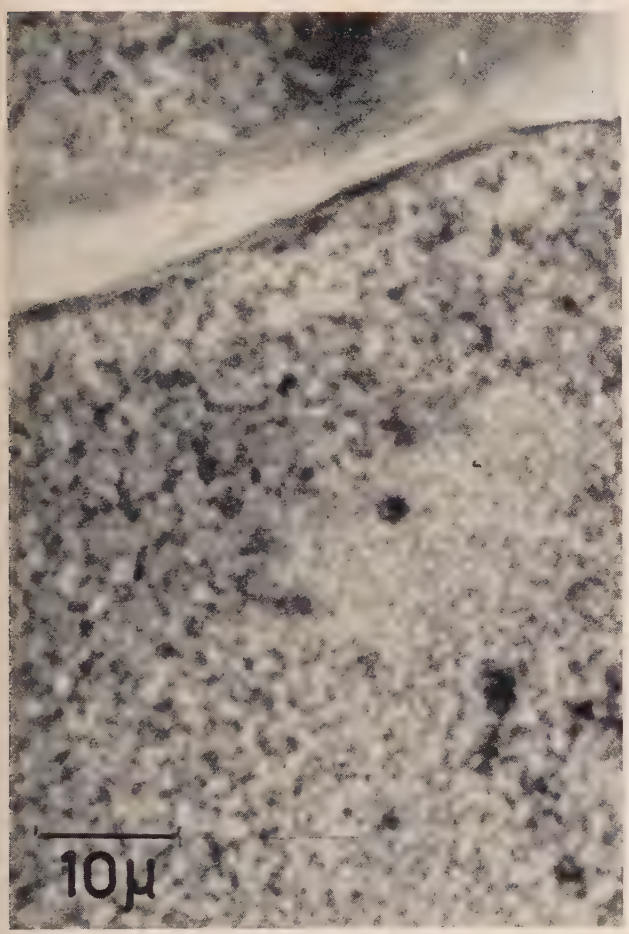


Fig. 1. Male pronucleus visible in a clear zone of an egg laid by an isolated individual (Feulgen, light green).

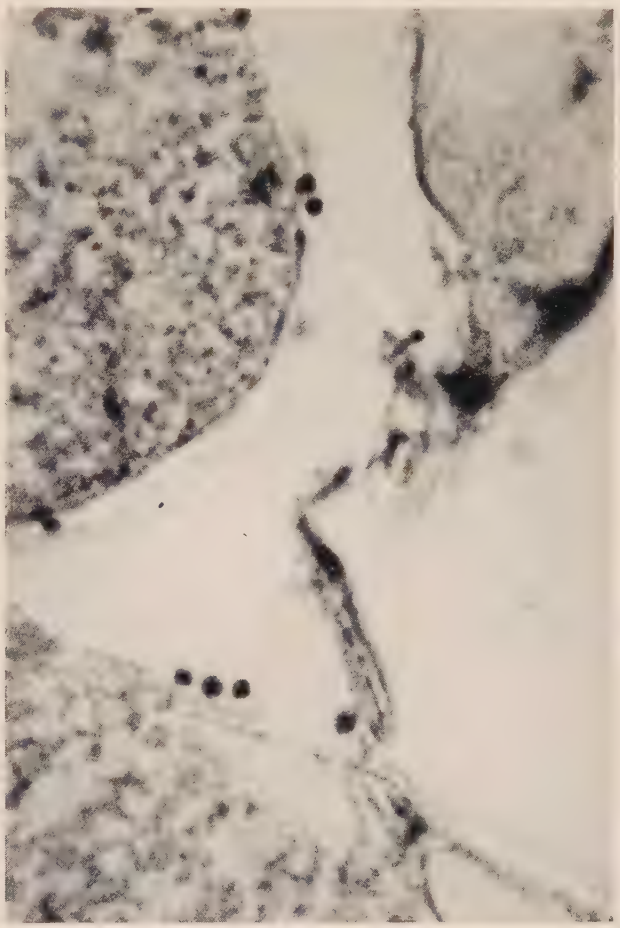


Fig. 2. Groups of sperms in ripe female phase individuals (Iron haematoxilin).

The culture methods used for *O. puerilis*[5] have been employed also for this new species and strains from individuals collected at the Leghorn Aquarium have been studied[6].

The following results were obtained: isolated individuals, kept in Boveri dishes with filtered sea-water, laid eggs that developed normally and gave rise to normal larvae after 8–10 days of development. This result suggested the possibility of parthenogenetic development which has already been observed in *Grubea pusilla*, another Polychaete[7].

[1] G. Bacci and M. La Greca, Boll. Zool. *27*, in print (1960).

[2] U. Parenti, R. C. Accad. Lincei *27*, in print (1960).

[3] G. Bacci *et al.*, Exper. *7*, 222 (1951); R. C. Accad. Lincei *28*, 92 (1960).

[4] G. Bacci and M. La Greca, Nature *171*, 1115 (1953).

[5] G. Bacci, Pubbl. Staz. zool. Napoli *26*, 110 (1955).

[6] The hearthiest thanks are due to Prof. Razzauti and to the staff of the Leghorn Aquarium.

[7] C. Hauenschild, Naturwissenschaften *42*, 611 (1955).

[8] G. Cognetti, Boll. Zool. *23*, 275 (1956); R. C. Accad. Lincei *24*, 325 (1958).

Older female phase individuals with ripe oocytes show again a few spermatocytes and sperms among oocytes which are ready to be laid. Sperms are always very scarce and they form small groups which are often adjacent to the oocytes (Fig. 2). Spermatocytes are generally found in clusters along the intestinal wall. The rapid secondary spermatogenesis is thus responsible for the subsequent self-fertilisation in *O. labronica*.

Similar processes were demonstrated by Cognetti in the Asteroid species *Asterina pancerii*[8].

Researches are now being carried on in order to ascertain whether primary and secondary spermatogenesis takes place in all individuals of *O. labronica*.

U. Parenti

Istituto di Zoologia, Università di Modena, and Acquario di Livorno (Italy), June 16, 1960.

Riassunto

Ophryotrocha labronica (Bacci e La Greca)[1] è ermafrodita proterandrica e si dimostra che molti individui presentano una fugace spermatogenesi secondaria che rende possibile l'autofecondazione.

Isolierung von Lysergsäure-Alkaloiden aus der mexikanischen Zauberdroge Ololiuqui (*Rivea corymbosa* (L.) Hall. f.)

Die Samen von *Rivea corymbosa* (L.) Hall. f., der alten aztekischen Zauberdroge «Ololiuqui»[1] und von *Ipomoea tricolor Cav.* (*Convolvulaceae*) werden auch heute noch von manchen Indianerstämmen Südmexikos für magische Zwecke verwendet.

R. G. Wasson verdanken wir Muster dieser beiden Drogen, die in Oaxaca (Mexiko) gesammelt worden waren. Durch schonende Extraktion konnte aus beiden Samen Alkaloidfraktionen gewonnen werden, die im Dünnschicht-Chromatogramm mindestens 5 übereinstimmende Indolverbindungen erkennen liessen. Es gelang, die 3 Hauptkomponenten zu kristallisieren und chemisch zu identifizieren.

Substanz A: Aus Methanol kristallösungsmittelfreie Prismen, Smp. 232–240° (korr.) Zers., $[\alpha]_D^{20} = +105°$ ($\pm 7°$) ($c = 0{,}1$ in Chloroform/Methanol 1 : 1). Gefunden: C 71,8; H 6,6; O 6,2; N 15,5%. Berechnet für $C_{16}H_{17}ON_3$ (267,3): C 71,9; H 6,4; O 6,0; N 15,7%. UV-Spektrum: Maxima bei 240 mμ ($\log \varepsilon_{max} = 4{,}3$) und bei 311 mμ ($\log \varepsilon_{max} = 3{,}9$). Dieses Spektrum ist mit demjenigen der Lysergsäure und Isolysergsäure praktisch identisch[2]. Diese physikalisch-chemischen und analytischen Daten entsprechen den Eigenschaften von d-Lysergsäure-amid. Die vollkommene Übereinstimmung der IR-Spektren von Substanz A und von d-Lysergsäure-amid (Iso-ergin)[3] bestätigt die Identität dieser beiden Verbindungen.

Substanz B: Aus Methanol kristallösungsmittelhaltige Prismen vom Smp. 136°(korr.), $[\alpha]_D^{20} = +383°$ ($\pm 6°$) ($c = 0{,}1$ in Chloroform/Methanol 1 : 1). Die im HV bei 120° getrocknete Verbindung lieferte folgende Analysenwerte: C 71,6; H 6,6; O 6,2; N 15,4%. Berechnet für $C_{16}H_{17}ON_3$ (267,3): C 71,9; H 6,4; O 6,0; N 15,7%. UV-Spektrum: identisch mit demjenigen von Substanz A. Diese Daten stimmten auf d-Isolysergsäure-amid (Ergin)[4]. Die Identität von Substanz B mit Ergin wurde durch die übereinstimmenden IR-Spektren bestätigt.

Substanz C: Aus Aceton massive kristallösungsfreie Polyeder vom Smp. 213–215° (korr.) Zers., $[\alpha]_D^{20} = -195°$ ($c = 0{,}2$ in Pyridin). Gefunden: C 74,6; H 7,9; O 6,5; N 11,0%. Berechnet für $C_{16}H_{20}ON_2$ (256,3): C 74,9; H 7,9; O 6,2; N 10,9%. UV-Spektrum: Maxima bei 222 mμ ($\log \varepsilon_{max} = 4{,}6$), bei 282 mμ ($\log \varepsilon_{max} = 3{,}9$) und bei 291 mμ ($\log \varepsilon_{max} = 3{,}8$). Diese Daten stimmten mit den Eigenschaften von Chanoclavin[5] überein. Die Identität von Substanz C mit diesem erstmals aus saprophytischen Kulturen des Mutterkornpilzes von *Pennisetum typhoideum* Rich. isolierten Alkaloid wurde durch den Vergleich der IR-Spektren, die vollkommene Übereinstimmung zeigten, bestätigt.

Das Vorkommen von Lysergsäure-Alkaloiden, die bisher nur in niederen Pilzen der Gattung *Claviceps* gefunden wurden, in der Pflanzenfamilie der *Convolvulaceae* ist ein unerwarteter, phytochemisch interessanter Befund.

Die Analysen wurden im mikroanalytischen Laboratorium SANDOZ (Leitung Dr. W. Schöniger) ausgeführt, die Spektren in der spektral-analytischen Abteilung (Leitung Dr. H. G. Leemann) aufgenommen. Für wertvolle Diskussion der Spektren danken wir Herrn Dr. H. G. Leemann bestens.

A. Hofmann und H. Tscherter

Pharmazeutisch-chemisches Laboratorium SANDOZ, Basel, 28. Juli 1960.

Summary

The psychotomimetic active principles of the ancient aztec drug «Ololiuqui» (*Rivea corymbosa* (L.) Hall. f.) have been found to be alkaloids of the ergot type. Three of the components could be crystallized and identified, i.e. ergine (isolysergic acid amide), isoergine (lysergic acid amide) and chanoclavin. The same alkaloids were found in the seeds of the related convolvulaceous vine *Ipomoea tricolor* Cav.

[1] Vgl. R. E. Schultes: *A contribution to our knowledge of Rivea corymbosa*. The narcotic ololiuqui of the Aztecs. Botanical Museum of Harvard University, Cambridge (Massachusetts) 1941.

[2] W. A. Jacobs, L. C. Craig und A. Rothen, Science *83*, 166 (1936).

[3] S. Smith and G. M. Timmis, J. Chem. Soc. *1936*, 1440.

[4] S. Smith and G. M. Timmis, J. Chem. Soc. *1932*, 764.

[5] A. Hofmann, R. Brunner, H. Kobel und A. Brack, Helv. Chim. Acta *40*, 1358 (1957).

Further Studies on Corticotropin Releasing Factor (CRF); Corticotropin Releasing Activity of Synthetic Peptides

In a preceeding communication[1] we have reported that it was possible to obtain, from hog posterior pituitary powder, a fraction free of vasopressin and yet having a strong CRF activity. By using the method of assay that we have described[1,2], we have now obtained peptide fractions of which 0.5–1.0 μg is as active as 4 μg of a standard preparation called CRF 91. These fractions have been obtained through the following operations: (1) Extraction of the posterior pituitary powder with dilute acetic acid; (2) Fractionated precipitation with acetone; (3) Countercurrent distribution according to the classical method of Craig; (4) Zone electrophoresis according to the method

[1] C. Gros and M. Privat de Garilhe, C. R. Acad. Sci., Paris *249*, 2234 (1959).

[2] M. Privat de Garilhe, C. Gros, J. Chauvet, C. Fromageot, C. Mialhe-Voloss, and J. Benoit, Biochim. biophys. Acta *29*, 603 (1958).

Substances	CRF-activity Versus 4 μg of CRF 91 [a]	MSH activity U/g
Natural		
Fraction 3	1.0 μg : 1.3	$5—6\times 10^{8}$ [6]
Fraction 4	1.0 μg : 1.3	
Fraction 5	1.0 μg : 0.8	
Synthetic		
N-ε-formyl-α-MSH [b]	4 μg : 0.7	2×10^{10} [9]
Hexapeptide [c]	4 μg : 1.2	2×10^{5} [6]
Heptapeptide	0.1 μg : 1.0	2.8×10^{5} [6]
Decapeptide	0.1 μg : 1.0	—

[a] As explained in ref. [1] and [2], one determines the amount of unknown substance, giving a release of ACTH equal to that given by 4 μg of the standard CRF 91: if we take for instance fraction 3, we see that 1 μg of this fraction provokes a release of ACTH equal to that produced by 1.3×4 μg of CRF 91, and that 0.1 μg of the heptapeptide produces a release equal to that given by 1.0×4 μg of CRF 91, etc. In the latter example, we would say that the heptapeptide is about 40 times more active than CRF 91; but this assumption can be made only when the ratio $x/4$ μg CRF 91 is equal to 1.0.

[b] N-ε-formyl-α-MSH: Ac.Ser.Tyr.Ser.Met.Glu.His.Phe.Arg.Try.Gly.Lys.Pro.Val(NH_2)
|
N-ε-formyl.

[c] Hexapeptide: NH_2
|
H.Glu.His.Phe.Arg.Try.Gly.OH

Heptapeptide: NH_2
|
H.Met.Glu.His.Phe.Arg.Try.Gly.OH

Deca-peptide: NH_2
|
H.Ser.Tyr.Ser.Met.Glu.His.Phe.Arg.Try.Gly.OH

of PORATH *et al.*[3]. The 3 first steps have already been described[1]; we describe here only the 4th step:

The material coming from the counter current distribution (25 mg) was dissolved in 1 ml of 0.1 *N* acid and put on a cellulose column of 0.6×25 cm; dinitrophenyl-ethanolamine was used as a tracer and the run was performed in 0.6 *M* pyridinium acetate; 0.2% phenol was added as a preservative and the temperature of the buffer was maintained at about 10–12°C with circulating water. The electrophoresis was run under 30 mA and 300 volts. Fractions of 2 ml were collected every 20 min and ninhydrin analysis was performed on 0.2 ml samples after acid hydrolysis (HCl 15 h in sealed tubes at 100°C).

In this manner several fractions were obtained numbered 1, 2, 3, 4, etc., the first one appearing after 12 h. The fraction 2 consists mainly of the tripeptide Leu. Arg. Leu; the CRF activity was found in peaks 3 and 4 (see Table). These 2 active fractions (3 and 4) represent approximately 10% of the material put on the column. Fractions 3 and 4 were also analyzed for their amino-acid composition: fraction 3 has a composition similar to that of the α-Melanophore Stimulating Hormone (α-MSH) given by HARRIS[4], with the exception that threonine and leucine were present.

The identity between α-MSH and CRF has been envisaged for a while by ourselves and by GUILLEMIN *et al.*[5]. This identity does not seem possible now for the following reasons:

– the presence of threonine and leucine in the molecule of the peptide(s) possessing CRF activity,
– the fact that the active fraction 3 has an MSH activity amounting only to 1–2.5% of that of pure α-MSH; this weak MSH activity may be either intrinsic to the material or may represent some contamination with α-MSH[6],
– the fact that highly purified natural α-MSH[7], as well as synthetic formyl-α-MSH, kindly supplied by Dr. K. HOFMANN, have no significant CRF activity.

In the course of these experiments we have studied several synthetic peptides related to the group of the Corticotropins and the Melanophore Stimulating Hormones, generously supplied by Drs. SCHWYZER and KAPPELER[8]. *We have found that several of these peptides, having a sequence in common with the Corticotropins and the MSH have a strong in vitro CRF activity, higher than that of the most active natural fractions so far obtained by us (Table).*

M. PRIVAT DE GARILHE, C. GROS,
J. PORATH, and EVA-BRITA LINDNER

Laboratoire de Chimie biologique de la Faculté des Sciences, Paris (France) and Biokemiska Institutionen, Uppsala (Sweden), June 1, 1960.

Résumé

Une fraction peptidique ayant une forte activité d'hypophyso-stimuline à effet corticotrope ou «Corticotropin Releasing Factor» (CRF), *in vitro*, a été obtenue à partir de la poudre d'hypophyses postérieures de porc par les opérations suivantes:

1. Extraction de la poudre avec l'acide acétique dilué; 2. Précipitation fractionnée à l'acétone; 3. Distribution à contre courant; 4. Electrophorèse de zones.

La constitution en amino acides de cette fraction est très voisine de celle de l'α-MSH; cependant α-MSH et CRF ne sont pas identiques pour des raisons chimiques et physiologiques. Bien que l'α-MSH n'ait pas d'activité CRF significative, des peptides synthétiques ayant une séquence commune avec l'α-MSH possèdent cette activité.

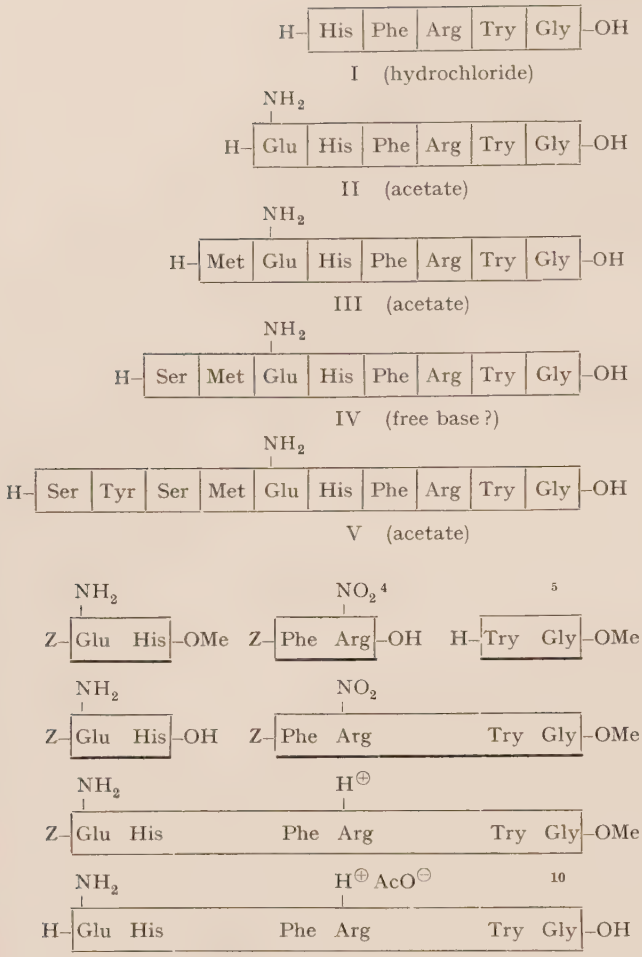
[3] J. PORATH, E. B. LINDNER, and S. JERSTEDT, Nature *182*, 794 (1958).
[4] J. I. HARRIS, Biochem. J. *71*, 451 (1959).
[5] R. GUILLEMIN, A. V. SCHALLY, and R. N. ANDERSEN, Fed. Proc. *19*, part I, 239 (1960).
[6] We are indebted to Dr. I. I. GESCHWIND (Berkeley) who was kind enough to perform MSH assays on this substance.
[7] A. V. SCHALLY, R. GUILLEMIN *et al.*, personal communication.
[8] H. KAPPELER and R. SCHWYZER, Exper. *16*, 183 (1960).
[9] K. HOFMANN, personal communication.

Synthetic Peptides Related to the Corticotropins (ACTH) and the Melanophore Stimulating Hormones (MSH) Possessing Corticotropin Releasing Activity (CRF-Activity)

Our synthetic work in the field of β-MSH[1] required the synthesis of a number of peptides with amino-acid sequences common to ACTH, α-MSH, and β-MSH. Among these were the pentapeptide (I), the hexapeptide (II), and the heptapeptide (III). The latter compound contains the whole amino-acid sequence common to all three hormones of the pituitary mentioned above.

LI[2] and HARRIS[3] have put forward the hypothesis that the common sequence might have some special biological

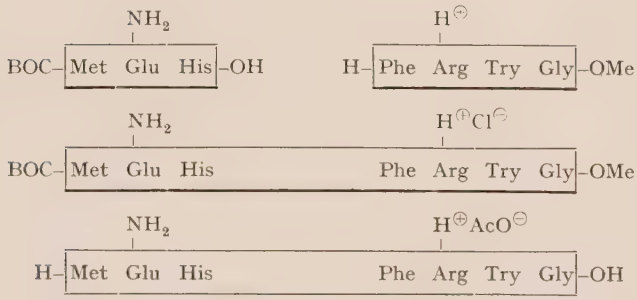
[1] R. SCHWYZER, H. KAPPELER, B. ISELIN, W. RITTEL, and H. ZUBER, Helv. chim. Acta *42*, 1702 (1959).
[2] C. H. LI, Adv. Protein Chem. *12*, 288 (1957).
[3] J. I. HARRIS and P. ROOS, Biochem. J. *71*, 434 (1959).


H– His Phe Arg Try Gly –OH
I (hydrochloride)
NH2
H– Glu His Phe Arg Try Gly –OH
II (acetate)
NH2
H– Met Glu His Phe Arg Try Gly –OH
III (acetate)
NH2
H– Ser Met Glu His Phe Arg Try Gly –OH
IV (free base ?)
NH2
H– Ser Tyr Ser Met Glu His Phe Arg Try Gly –OH
V (acetate)

II. amorphous, $[\alpha]_D^{26} = -23.6° \pm 1.8°$ ($c = 0.466$ in 1 *N* HCl); R_f $45^6 = 0.47$; R_f $43^{11} = 0.35$ (Whatman No. 3); Amino-acid analysis[7]: Glu_1, His_1, Phe_1, Arg_1, Gly_1, $NH_{3\,1.2}$ (tryptophane was destroyed during hydrolysis). Leucineaminopeptidase completely hydrolyses II within a short period of time.

Fig. 1. *Synthesis and properties of the hexapeptide II*

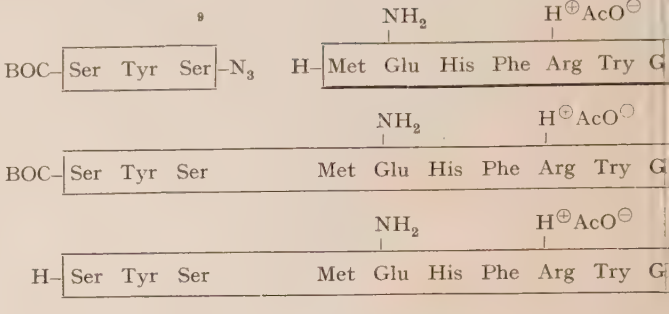
Z = carbobenzoxy. Strong underline denotes crystallinity of the compound. Condensations were performed with dicyclohexyl-carbodiimide[8].



III. Mp. 192°C (decomp.); $[\alpha]_D^{26} = -25.3° \pm 7°$ ($c = 1.108$ in dimethyl formamide); amino-acid analysis[12]): Met_1, Glu_1, His_1, Phe_1, Arg_1, Gly_1, $NH_{3\,1}$ (tryptophane was destroyed during hydrolysis).

Fig. 2. *Synthesis and Properties of the Heptapeptide III*

BOC- = *t*-Butyloxycarbonyl; condensation was performed with dicyclohexyl-carbodiimide[13].


9
NH2
H⊕AcO⊖
BOC– Ser Tyr Ser –N3 H– Met Glu His Phe Arg Try G
NH2
H⊕AcO⊖
BOC– Ser Tyr Ser Met Glu His Phe Arg Try G
NH2
H⊕AcO⊖
H– Ser Tyr Ser Met Glu His Phe Arg Try G

V: amorphous, $R_f 45^{11} = 0.51$, $R_f 50^{10} = 0.47$, $R_f 54^{15} = 0.58$

Fig. 3. *Synthesis and properties of the decapeptide V*

significance, especially with respect to the melanophore stimulating properties of all three types of hormone. The peptides I, II, and III actually do elicit a very small MSH-activity which increases with the length of the peptide chain (I = 0.3×10^5, II = 2×10^5, and III = 2.8×10^5 U/g in the *in vitro* assay[11]), compared with α-MSH = 2×10^{10}, βb-MSH = 2×10^9 and αb-ACTH = 0.5×10^8 U/g). Hofmann *et al.* had already synthesized I as well as the octapeptide IV, the latter being active at a level of 7×10^5 U/g [12]. The heptapeptide III displays no ACTH-activity in the Sayers' assay[13] as found by Prof. Dr. W. Schuler in our biological laboratories.

Some of our synthetic peptides were kindly tested for CRF-activity at the Laboratoire de Chimie Biologique of the University of Paris with the *in vitro* assay of Saffran, Schally, and Benfey[14]. Dr. M. P. de Garilhe and Mlle Cl. Gros found that especially the heptapeptide (III) has a very high activity as compared with isolated, natural material. As the hexapeptide (II) was markedly less potent, we prepared the decapeptide (V): it proved to be active in the same range as III. These are the first synthetic peptides ever reported to have CRF-activity.

De Garilhe, Gros, Porath, and Lindner are simultaneously communicating the biological aspects of these

[4] Paper chromatography system No. 43 is: *t*-amylic alcohol/*iso*-propanol/water (100:40:55); system No. 45 is: *sec.*-butanol/3% solution of ammonia (100:44).

[5] S. Moore, D. H. Spackman, and W. H. Stein, Analyt. Chem. *30*, 1185, 1190 (1958). Our thanks are due to Prof. Dr. M. Brenner and Dr. R. Weber (Org.-chemische Anstalt der Universität Basel) for kindly doing the analyses.

[6] J. C. Sheehan and G. P. Hess, J. Amer. chem. Soc. *77*, 1067 (1955).

[7] Kindly furnished by Dr. B. Iselin of our Research Division.

[8] Paper chromatography system No. 50 is: *t*-amyl alcohol/isopropanol/triethylamine/veronal/water (100 ml:40 ml:0.8 ml:1.8 g: 50 ml); system No. 54 is: *sec.*-butanol/isopropanol/monochloroacetic acid/water (70 ml:10 ml:3 g:40 ml).

[9] We wish to thank Dr. C. H. Li, Hormone Research Institute, U. C., in Berkeley (California), for carrying out the assays.

[10] K. Hofmann, M. E. Woolner, G. Spühler, and E. T. Schwartz, J. Amer. chem. Soc. *80*, 1486 (1958). – K. Hofmann, Th. A. Thompson, and E. T. Schwartz, J. Amer. chem. Soc. *79*, 6087 (1957).

[11] M. A. Sayers, G. Sayers, and C. A. Woodbury, Endocrinology *42*, 379 (1948).

[12] M. Saffran, A. V. Schally, and B. G. Benfey, Endocrinology *57*, 399 (1957).

[13] M. P. de Garilhe, Cl. Gros, J. Porath, and E. B. Lindner, Exper. *16*, 414 (1960).

[14] K. Hofmann, W. Peckham, and A. Rheiner, J. Amer. chem. Soc. *78*, 238 (1956).

[15] H. Kappeler, detailed paper for Helv. chim. Acta in preparation.

findings[15]. We shall here only give a preliminary report on the syntheses of the compounds II, III, and V (Fig. 1–3); detailed papers are to appear elsewhere.

H. Kappeler and R. Schwyzer

Forschungslaboratorien der CIBA Aktiengesellschaft, Pharmazeutische Abteilung, Basel, June 1, 1960.

Zusammenfassung

Einige Peptide, strukturell ähnlich den gemeinsamen Aminosäuresequenzen der drei Hypophysenhormone ACTH, α- und β-MSH, wurden synthetisiert. Sie zeigen eine sehr schwache Wirkung auf die Melanophoren (V wurde nicht geprüft); das Heptapeptid (III) besitzt keine ACTH-Aktivität. Die drei Verbindungen II, III und V vergrössern die ACTH-Ausschüttung im *in vitro*-Versuch von Saffran, Schally und Benfey[7] stark. Damit sind die ersten synthetischen Peptide mit dieser CRF-Aktivität gefunden.

Adrenaline-Noradrenaline Content of the Submaxillary Gland of the Cat

It has been suggested that some sympathicomimetic amines, e. g. tyramine, exert their effect by release of adrenaline-noradrenaline (Burn and Rand[1]). In the present experiments, the effect on the adrenaline-noradrenaline content of the submaxillary gland caused by infusion of tyramine has been studied.

Methods. Ten cats were used for the experiments. One series consisted of three normal cats. In a series of five cats, the effect of an intravenous infusion of tyramine was studied. In two cats, the superior cervical ganglion of one side was excised 3 weeks before the experiment.

In the acute experiment, the cats were given chloralose (80 mg/kg) after preliminary ether anesthesia. To facilitate a quick removal, the glands were dissected, leaving nerves, vessels, and secretory duct intact. The excised gland was immediately put into a cooled homogenizer containing 10 cm^3 of ice – chilled 0.4 *N* perchloric acid. The homogenate was kept in a deep-freeze (– 20°C) until the estimation of the catechols was made one or two days later.

Estimation of adrenaline-noradrenaline[2]. The noradrenaline and adrenaline content of the homogenates was estimated according to the fluorometric method described by Bertler, Carlsson, and Rosengren[3]. The simplified extraction variant of the procedure was used. A slight error is admittedly introduced hereby, especially since the glands were not weighed, in order to reduce the time delay between removal and homogenization. The error introduced is, however, only of the order of 1%, even if it is assumed that the weight of an organ weighing about 1 g can be estimated by sight not better than within ± 0.5 g. The elution was made with 8 ml 1 *N* HCl and the estimation made on 2 ml of the eluate.

Further technical details are given below.

Results. The normal submaxillary gland was found to contain only small amounts of adrenaline in comparison with the amounts of noradrenaline. The two normal glands from a single animal were very similar in their noradrenaline content (Table), and therefore the effect of tyramine infusion or of ganglionectomy on the noradrenaline content could be studied on pairs of glands, using one gland as a control.

Infusion of tyramine was started after one gland had been removed and homogenized. A cannula was tied into the secretory duct of the remaining gland. Each animal was then given 150 mg of tyramine intravenously during approximately 1 h. At a constant rate of infusion, the secretory rate declines. In order to keep up a secretion the rate of infusion was increased intermittently. In spite of this, the gland can not be made to secrete for a long time. At the end of infusion, when the gland was removed, there was no secretion in any of the cases even though huge doses were given (up to 10 mg/min).

The infusion was found to lower the noradrenaline content of the gland to some 50% (Table).

Excision of the superior cervical ganglion caused a complete disappearence of noradrenaline, while adrenaline was found in seemingly normal quantities (Table).

Expressed in μg, the noradrenaline and, within brackets, the adrenaline content of the submaxillary gland of the cat.

Cat. No.	Procedure	Right gland	Left gland
1	Normal glands	0.71 (0.04)	0.71 (0.10)
2		0.78 (0)	0.74 (0.06)
3		0.69 (0)	0.66 (0)
		Mean value: 0.73 (0.01)	0.70 (0.05)
4	Right superior cervical ganglion excised 3	0 (0.06)	0.45 (0.07)
5	weeks earlier	0 (0.06)	1.24 (0.06)
		Mean value: 0 (0.06)	0.85 (0.07)
6	Left gland removed before and right gland	0.49 (0.06)	1.16 (0.04)
7	removed after infusion of tyramine	0.59 (0.02)	1.29 (0)
8		0.51 (0.02)	0.85 (0)
9		0.40 (0.07)	1.01 (0)
10		0.48 (0.05)	0.91 (0)
		Mean value: 0.49 (0.04)	1.04 (0.01)

[1] J. H. Burn and M. J. Rand, J. Physiol. *144*, 314 (1958).

[2] I am greatly indebted to Drs. Å. Bertler, A. Carlsson, and E. Rosengren for facilities to make the estimations.

[3] Å. Bertler, A. Carlsson, and E. Rosengren, Acta physiol. scand. *44*, 273 (1958).

[4] R. Strömblad, Acta physiol. scand. *36*, 154 (1956).

[5] B. C. R. Strömblad, Brit. J. Pharmacol., in press.

[6] N. Emmelin and J. Engström, J. Physiol., in press.

Discussion. The decrease in the noradrenaline content of the submaxillary gland following an infusion of tyramine supports the hypothesis that tyramine causes a release of noradrenaline. It may therefore well be that the effects of tyramine are exerted via a release of noradrenaline. The total disappearence of noradrenaline from the gland, as well as of the secretory response to tyramine (Strömblad[4,5]) after removal of the sympathetic supply, is also consistent with such a mode of action of tyramine.

At the end of the infusion of tyramine, no secretory response was obtained, but the gland was found to retain some 50% of its noradrenaline. No explanation can be given; but it could be pointed out that the sympathetic innervates also the vessels of the gland and therefore the remaining noradrenaline could be associated with terminals around the vessels. On electrical stimulation of the sympathetic, the nerve terminals in contact with the secretory cells seem to be more easily depleted of transmitter than those in contact with the vessels (Emmelin and Engström[6]).

The figures for the adrenaline content of the glands were low and varied much more than those for the noradrenaline content. It is, however, of interest to note that adrenaline was present in apparently normal quantities after excision of the superior cervical ganglion. It could be that adrenaline is located outside the nerve terminals in the submaxillary gland.

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Institute of Physiology, University of Lund (Sweden), May 27, 1960.

Zusammenfassung

Der Noradrenalingehalt in der Submaxillarisdrüse der Katze nahm nach der Infusion von Tyramin ab, wodurch die Auffassung unterstützt wird, dass diese Substanz durch Freisetzung von Noradrenalin wirkt. Sympathische Ganglionektomie verursachte das vollständige Verschwinden von Noradrenalin in der Drüse.

In Vivo Uptake of Dopamine and 5-Hydroxytryptamine by Adrenal Medullary Granules

In 1953 the adrenaline and noradrenaline of the adrenal medulla were shown to be localized within intracellular granules of about the same size as mitochondria but not identical with these[1,2]. Subsequent observations[3,4] indicate that also catechol amines in other tissues are kept in similar particles. In studies of this storage mechanism, the adrenal medulla is often the tissue of choice, because it is easy to isolate and prepare. However, it has the drawback of a comparably slow turnover, which makes investigations of the kinetics of the storage mechanism difficult.

In an attempt to overcome the difficulty, we have studied the uptake of catechol amines of the catechol amine containing granules after administration of 3,4-dihydroxyphenylalanine (DOPA). This amino acid penetrates into the cell, where it is decarboxylated to dopamine by means of DOPA-decarboxylase. This enzyme is located in the cytoplasmic sap[5]. It was observed that most of the newformed dopamine was present in the granular fraction after differential centrifugation. The procedure is briefly described below.

L-DOPA in a dose of 100 mg/kg was administered i. v. into rabbits weighing about 2 kg. The animals were killed at varying intervals of time after the injection. The adrenals were rapidly removed, and the adrenal medulla was dissected free from cortical tissue. The medulla was homogenized in ice-cooled 0.3 *M* sucrose in a Potter-Elvehjem homogenizer. From the homogenate three fractions were prepared by means of differential centrifugation according to Hillarp *et al.*[1]: (1) the low-speed sediment, containing cell nuclei and cell debris, (2) the high speed sediment, containing the mitochondria and catechol amine storing granules, (3) the high speed supernatant, containing the cytoplasmic sap. The catechol amine contents of the fractions were determined as previously described[6]. As will be seen in the Table, the granular fraction contained 3.7 μg dopamine 30 min after the administration of DOPA, whereas less than 1 μg was localized in the cytoplasmic sap. The newly-formed dopamine is thus rapidly taken up by the granules. In the kidney cortex, however, which is rich in DOPA-decarboxylase but lacks catechol amine storing granules, most of the dopamine formed was found in the cytoplasmic sap. Pretreatment of the animals with reserpine impaired the uptake of dopamine by adrenal medullary granules after DOPA administration. Thus the primary effect of reserpine which is known to deplete the body stores of catechol amines[7–9] may be its interaction with the uptake of catechol amines by the granules.

In-vivo uptake of dopamine and 5-hydroxytryptamine by adrenal medullary granules after administration of DOPA and 5-hydroxytryptophan respectively (μg per pair of adrenals. Each value represents the mean of two determinations. DA: dopamine; NA: noradrenaline; A: adrenaline; 5-HT: 5-hydroxytryptamine; 5-HTP: 5-hydroxytryptophan).

		DA	NA	A	5-HT
DOPA, 100 mg/kg (30 min)	Cytoplasmic sap	0.8	0.9	8.2	—
	Granules	3.7	4.8	72	—
DOPA, 100 mg/kg (1 h)	Cytoplasmic sap	1.4	2.7	14.5	—
	Granules	5.4	15.5	127	—
Reserpine, 5 mg/kg (3 h) + DOPA, 100 mg/kg (30 min)	Cytoplasmic sap	1.5	2.0	11.6	—
	Granules	2.0	–0.5	69	—
Reserpine, 5 mg/kg (20 h) + DOPA, (30 min)	Cytoplasmic sap	4.0	1.3	1.1	—
	Granules	0.8	1.0	1.9	—
5-HTP, 100 mg/kg (1 h)	Cytoplasmic sap	—	—	—	0.45
	Granules	—	—	—	1.40
Control	Cytoplasmic sap	0.0	0.2	6.7	0.0
	Granules	0.3	1.2	105.3	0.0

[1] N.-Å. Hillarp, S. Lagerstedt, and B. Nilsson, Acta physiol. scand. *29*, 251 (1953).

[2] H. Blaschko and A. D. Welch, Arch. exp. Path. Pharmak. *219*, 17 (1953).

[3] U. S. v. Euler and N.-Å. Hillarp, Nature *177*, 44 (1956).

[4] Å. Bertler, N.-Å. Hillarp, and E. Rosengren, Acta physiol. scand., in press.

[5] H. Blaschko, P. Hagen, and A. D. Welch, J. Physiol. *129*, 27 (1955).

[6] Å. Bertler, A. Carlsson, E. Rosengren, and B. Waldeck, Kgl. Fysiograf. Sällskap. Lund Förh. *28*, no. 12 (1958).

[7] A. Carlsson and N.-Å. Hillarp, Kgl. Fysiograf. Sällskap. Lund Förh. *26*, no. 8 (1956).

[8] Å. Bertler, A. Carlsson, and E. Rosengren, Naturwissenschaften *22*, 521 (1956).

[9] A. Carlsson, E. Rosengren, Å. Bertler, and J. Nilsson, *Psychotropic Drugs* (S. Garattini and V. Ghetti, Eds. Elsevier Publishing Company, Amsterdam 1957), p. 363.

[10] Å. Bertler and E. Rosengreen, Exper. *15*, 382 (1959).

[11] E. Rosengren, Acta physiol. scand., in press.

The mechanism by which catechol amines are taken up and stored does not seem to be specific for catechol amines, 5-hydroxytryptamine (5-HT) being found in the catechol amine containing granules after administration of DL-5-hydroxytryptophan (5-HTP) (Table).

From these experiments it thus may be concluded that 5-HT and catechol amines are stored in the cells in similar particles, the identity of which is under further investigation. Which of the two types of amines a cell containing these granules possesses, seems to be due to its contents of DOPA and 5-HTP synthezising enzymes, as 5-HTP decarboxylase appears to be identical with DOPA-decarboxylase[10,11].

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Department of Pharmacology, University of Lund (Sweden), May 9, 1960.

Zusammenfassung

Die Einlagerung von neugebildetem Dopamin in den Katecholamin-haltigen Granula des Nebennierenmarks nach Injektion von DOPA ist untersucht worden. Dopamin wird von den Granula aufgenommen, und nur wenig davon wird im Cytoplasma gefunden. Vorbehandlung der Tiere mit Reserpin verhindert diese Einlagerung. Der Mechanismus der Einlagerung ist nicht spezifisch, weil 5-Hydroxytryptamin nach Injektion von 5-Hydroxytryptophan von den Granula auch aufgenommen wird.

Storage of New-Formed Catecholamines in the Adrenal Medulla

In previous papers[1,2] it has been shown that large amounts of dopamine (DA) and noradrenaline (NA) are rapidly formed in the adrenaline (A) cells of the rabbit suprarenal medulla after an intravenous injection of L-3,4-dihydroxyphenylalanine (dopa). It was also found that the new-formed amines rapidly become 'particle-bound'. In the present study, evidence is presented in support of the view that DA and NA are in fact incorporated in the amine granules which normally store only A.

In three experiments, the intracellular distribution of DA, NA, and A was examined by means of density gradient centrifugation. In each experiment, the adrenal medullas from two rabbits (1.5 to 2 kg), to which dopa (100 mg/kg body weight) had been given 1 h previously, were homogenized in 0.3 *M* sucrose and then fractionated into a low-speed sediment (800 × *g*, 5 min), a 'large granule' fraction and a high-speed supernatant (20000 × *g*, 20 min; details are found in a previous paper[2]). The 'large granule' fraction, i. a. containing the mitochondria and the amine storage granules, was resuspended in sucrose and subjected to density gradient centrifugation (gradient: 0.4 to 1.8 *M* sucrose) in the way described previously[3]). The content of the tube was divided into three subfractions: a top fraction (G1) containing microsomal material, mitochondria, and amine granules of low density, a bottom fraction of non-sedimented amine granules of high density (G2), and a small sediment of high density amine granules which seem to be practically free from other cell particles[3]. The amine content in all the fractions was determined spectrophotofluorimetrically[4,5].

It was found (Table I) that the 'particle-bound' DA and NA showed practically the same distribution as that of A. Most noteworthy is the fact that 50 to 60% of all three amines were recovered in the sediment (G3) containing the high density amine granules. These findings strongly support the view that the new-formed DA and NA must have been taken up in the specific amine storage granules. It seems quite unlikely that the medullary cell contains any other 'particles' that - besides being able to bind the large amounts of DA and NA – have the same sedimentation characteristics as those of the storage granules.

The newly formed DA and NA were not taken up preferentially by the amine granules of low density. This is interesting since these granules which – in contrast to the high density granules – seem to store amines largely without adenosinetriphosphate might be thought to represent granules in an early storage phase[6]. The present findings do not support such a view.

Another approach to the storage problem was also attempted. There are several findings suggesting that reserpine destroys the amine storage mechanism[7,8]. It is thus of interest to see whether the DA and NA formed after a dopa injection become 'particle-bound' also in animals treated with reserpine.

Reserpine (5 mg/kg body weight) was administered to rabbits and dopa was injected after varying periods of time (Table II). After homogenization of the medullas

Tab. I

Exp. Nr.	Amine	Low-speed sediment µg	High-speed supernatant µg	Granule-bound amines Total µg	Distrib. in % of total G1	G2	G3
I	A	13	15	255	11	34	55
	NA	3.2	3.1	42	9	31	59
	DA	0.8	0.8	10	11	32	57
II	A	26	15	260	10	31	60
	NA	−0.1	1.6	55	10	36	54
	DA	1.0	0.5	9.2	12	28	60
III	A	15	23	175	6	45	49
	NA	2.1	0.6	18	13	35	52
	DA	0.7	1.9	4.3	7	42	51

Tab. II

	Adrenaline Total µg	Adrenaline 'Free' %			Noradrenaline Total µg	Noradrenaline 'Free' %			Dopamine Total µg	Dopamine 'Free' %		
Reserpine 3 h Dopa 30 min	91	83	12	17	1.8	1.0	100	100	3.8	2.9	42	44
Reserpine 5 h Dopa 30 min	37	24	24	18	4.4	1.9	54	65	4.0	2.3	49	57
Reserpine 20 h Dopa 30 min	3.6	3.3	21	57	1.9	3.3	53	57	5.0	5.7	82	80

[1] Å. Bertler, N.-Å. Hillarp, and E. Rosengren, Ciba Found. Symp. Adrenergic Mechanisms. J. & A. Churchill, London, in press (1960).

[2] Å. Bertler, N.-Å. Hillarp, and E. Rosengren, Acta physiol. scand., in press (1960).

[3] N.-Å. Hillarp, Acta physiol. scand. *43*, 82 (1958).

[4] A. Carlsson and B. Waldeck, Acta physiol. scand. *44*, 293 (1958).

[5] Å. Bertler, A. Carlsson, and E. Rosengren, Acta physiol. scand. *44*, 273 (1958).

[6] N.-Å. Hillarp, Acta physiol. scand., in press (1960).

[7] A. Carlsson, E. Rosengren, Å. Bertler, and J. Nilsson, in *Psychotropic Drugs* (Ed.: S. Garattini and V. Ghetti, Elsevier Publ. Comp., Amsterdam 1957), p. 363.

[8] F. B. Hughes and B. B. Brodie, J. Pharmacol. exp. Therap. *127*, 96 (1959).

(one animal in each experiment), the low-speed sediment and the high-speed supernatant and sediment were examined. The experiments clearly showed that to a high extent reserpine may prevent the newly formed DA and NA from becoming 'particle-bound'. This result thus further supports the conclusion that these amines – after dopa administration to normal animals – are incorporated in the specific storage granules.

The findings in the present study, together with those in a previous work[2], seem to admit of the following general conclusions.

(1) The incorporation of newly formed amines in the storage granules is a rapid process.

(2) The storage mechanism is highly efficient since the newly formed amines are rapidly taken up by the granules, even at very high rates of synthesis.

(3) The storage mechanism is non-specific in the sense that it cannot choose between DA, NA, and A.

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Zusammenfassung

Die intrazellulare Lokalisation des Dopamins und Noradrenalins, die schnell nach einer intravenösen Injektion von L-DOPA in die Adrenalinzellen des Nebennierenmarks gebildet werden, ist bei normalen und reserpinbehandelten Kaninchen untersucht worden. Die Versuche sprechen dafür, dass die neugebildeten Amine sehr schnell den spezifischen Granula, die gewöhnlich nur Adrenalin enthalten, einverleibt werden. Der Einlagerungsmechanismus der Amine scheint sehr wirkungsvoll zu sein, aber unspezifisch in dem Sinn, dass er nicht zwischen Dopamin, Noradrenalin und Adrenalin unterscheidet.

Immunological Tolerance in Chickens Induced by Blood Group Substance

Since the classical work of Billingham *et al.*[1], numerous investigators have found that the treatment of embryos, and newborn or newly hatched animals, with foreign red blood cells or proteins may be followed by immunological tolerance. Some workers[2] have succeeded in producing in chickens an immunological tolerance to erythrocytes. On the other hand, Baer *et al.*[3] have shown that chickens injected with hog blood group substance are able to produce the agglutinins which react with human red cells. However, there are no data in the literature concerning the induction in animals of immunological tolerance to human erythrocytes by means of blood group substances. This is the subject of this preliminary communication, which represents only a part of a larger work.

The 12-day old Rhode Island Red embryos and infant chickens from the 2nd to the 32nd day of age were injected with hog 0(H) blood group substance (kindly provided by Dr. E. A. Kabat, Department of Microbiology, Columbia University, N. Y). The embryos received intravenously 0·08 mg of blood group substance, and the treatment was followed during posthatched life until the 32nd day of age (subcutaneous injections of 0·08 mg of substance each in three-day intervals). The challenging injections of human group 0 red cells were performed on the 42nd and 62nd day of postembryonic life. The control group of chickens was not preliminary treated with hog 0(H) substance, and received two provocative injections of 0 red cells at the age of 42 and 62 days. The treated and control chickens were bled on the 50th and 70th day of age. The haemagglutination reaction was performed in small 7 × 45 mm tubes, using serial doubling dilutions of chicken sera starting from 1:10, and 1% suspension of human 0 erythrocytes. The incubation lasted 2 h at room temperature and the reactions were read microscopically. The hemagglutinin titers were expressed in the term of logarithms to the base 2.

The first and second antibody responses of chickens to the injected human group 0 red cells are presented in the Table. The data clearly show that hog 0(H) blood group substance, used for the treatment of chickens in early life, may elicit the depression in the formation of agglutinins reacting with human 0 red cells. It is of interest to note that an antigen which is chemically a muco-polysaccharide, mainly composed of non-reducing and reducing carbohydrates, represents an active substance able to produce the immunological tolerance in chickens to human erythrocytes. Furthermore, the foregoing data indicate that an antigen from one animal species (hog), when injected into another species (chicken), may cause immunological tolerance of red cells belonging to a third animal species (man). This phenomenon is based upon the same serological specificity of hog 0(H) substance and human group 0 red cells.

Chicken	Normal haemagglutinins 10 chickens Age: 42 days MPT ± SD	First response 10 chickens Age: 50 days MPT ± SD	Second response 10 chickens Age: 70 days MPT ± SD
Treated with hog 0 (H) substance	4·02 ± 0·69	6·52 ± 1·11	7·98 ± 0·81
Control	3·62 ± 0·45	8·42 ± 0·94	10·72 ± 0·49

MPT: geometric mean peak agglutinin titer (log_2).
SD: standard deviation.

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Microbiological Institute, Faculty of Pharmacy, Belgrade University, Belgrade (Yugoslavia), December 7, 1959.

Résumé

L'injection de substance 0(H) du porc à des embryons et à des poussins récemment éclos a comme résultat un abaissement de leur capacité de produire dorénavant l'immunisation active d'agglutinines spécifiques pour les globules rouges humains du groupe 0.

[1] R. E. Billingham, L. Brent, and P. B. Medawar, Nature *172*, 603 (1953).

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The Influence of Estradiol, Progesterone, and Relaxin on the Peroxidase Activity in the Uterus

During earlier investigations[1,2], it was noticed that the peroxidase activity in rat uterus[3,4] varied considerably from one animal to another and also that the pattern depended upon the test method. The peroxidase activity of the pregnant uterus and the effects of estradiol, progesterone, and relaxin on the apparent peroxidase activity in the non-pregnant uterus have therefore been studied.

Material and methods. White mice weighing 24–26 g were used. The uterus was homogenized with water (equal results with 150 *mM* KCl), 1 ml/100 mg, and debris removed by glass wool. The extracts were assayed for nitrogen with the micro-Kjeldahl procedure and for peroxidase with guaiacol[5] at pH 7·1 and 4·9 (GP) and with mesidine[6] at pH 4·9 (MP). The activity is expressed as the inverse of the time needed for a given increase in absorbance, 0·050 with guaiacol and 0·020 with mesidine. A Beckman DU spectrophotometer and 3 ml cells were used.

Six spayed animals were given 1·5 μg estradiol cyclopentyl propionate[7] subcutaneously once five days prior to sacrifice. Another six animals received in addition 2 mg relaxin[8] in 20% polyvinylpyrrolidone[9] 6 h before sacrifice. In a third series, estradiol benzoate[10] (1 μg subcutaneously per day × 3), progesterone[11] (2 mg subcutaneously per day × 3), and relaxin (single subcutaneous injection 6 h before sacrifice) were given as shown in Table I.

The pregnant mice were sacrificed on day 18–20 of gestation.

Tab. I. 'Guaiacol peroxidase' activity in homogenates of uteri from spayed mice, given various combinations of estradiol, progesterone, and relaxin (mean ± SD).

Treatment	Number of animals	Wet weight mg	Nitrogen in extract mg/ml	Activity $1/t_{0.05}$ min^{-1} mg N^{-1}
Estradiol[7]	6[c]	79±8	1·16 ± 0·14	195 ± 63[a]
Estradiol[7] + relaxin	6[c]	109±11	1·20 ± 0·04	166 ± 76[a]
Peanut oil + relaxin	1	20	1·54	0[a]
Estradiol[10]	5	83±6	0·91 ± 0·07	14·2 ± 4·4[b]
Estradiol[10] + progesterone	5	45±1	Not det.	0[b]
Estradiol[10] + relaxin	4	119±21	0·82 ± 0·12	10·4 ± 1·4[b]
Estradiol[10] + progesterone + relaxin	4	62±8	Not det.	0[b]
Peanut oil	4	21±1	Not det.	0[b]

[a] Assayed at pH 7·1. [b] Assayed at pH 4·9. [c] Assayed in pairs.

Results. GP was found in the uterus from spayed animals given estradiol, but the attempts to demonstrate MP in the same homogenates gave negative results (0·2 ml). Only occasionally and after several minutes was a faint pink colour seen in the mesidine test. Controls showed that GP retained part of its activity at the pH and the peroxide concentration used for MP. Extracts from animals given estradiol + progesterone oxidized neither guaiacol nor mesidine. No change in the peroxidase pattern was brought about by relaxin under our experimental conditions.

The MP reaction with homogenates from pregnant uteri was consistently much more intensive than the faint one occasionally seen in estradiol-treated animals (Table II), but the test for GP was consistently negative (0·2 ml, pH 7·1 and 4·9). The MP activity was not caused by a local effect of the placenta.

Tab. II. 'Mesidine peroxidase' homogenates of uteri from pregnant mice. Two organs were used for every homogenate, and 0·05 ml taken for the assay.

Tissue	Nitrogen in homogenate mg/ml	Activity $1/t_{0.020}$ min^{-1}mg N^{-1}
Bilateral pregnancy:		
Whole uterus	0·85	6·5
	0·67	6·3
Placental insertions	1·18	11·5
Residual tissue	1·41	6·9
Unilateral pregnancy:		
Pregnant horns	1·11	10·8
	1·25	9·6
Non-pregnant horns	0·81	10·0
	1·05	6·6

Both GP and MP activities are peroxidatic in nature since no colour appeared unless hydrogen peroxide was added, but there seems to be some reciprocity in their occurrence. GP may be identical with the peroxidase of rapidly proliferating tissues[4]. The nature of MP is at present left open.

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K. G. Paul and N. Wiqvist

Biokemiska Institutionen and Wallenberglaboratoriet, Karolinska Institutet, Stockholm, and Kvinnokliniken, Karolinska sjukhuset, Stockholm (Sweden), October 19, 1959.

Zusammenfassung

Es wird gezeigt, dass die Uterusperoxydase aus zwei Komponenten besteht, die unter bestimmten Hormoneinwirkungen getrennt auftreten und mit verschiedenen Methoden nachweisbar sind.

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[2] G. Bloom and K. G. Paul, Exper. *15*, 22 (1959).

[3] F. V. Lucas, H. A. Neufeld, J. G. Utterback, A. P. Martin, and E. Stotz, J. biol. Chem. *214*, 775 (1955).

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[6] K. G. Paul and Y. Avi-Dor, Acta chem. scand. *8*, 649 (1954).

[7] ECP* (Upjohn).

[8] Prepared from pregnant sow's ovaries according to E. H. Frieden and F. L. Hisaw, Arch. Biochem. *29*, 166 (1950) and assaying approximately 30 GPU per mg. We are indepted to Dr. R. L. Kroc, Warner-Chilcott laboratories, for arranging this assay.

[9] Periston® (Bayer).

[10] Ovex® (Leo).

[11] Progestin® (Pharmacia).

Herstellung und biologische Wirkung des 16α-Methyl-desoxycorticosteron-acetats*

Über die Zusammenhänge zwischen chemischer Struktur und biologischen Wirkungen einiger Sterole wurde vor einiger Zeit referiert[1,2]. Insbesondere wurden damals von uns die biologischen Folgen der 1,2-Dehydrierung und der 14α-Hydroxylierung bei einigen, schwach glucocorticoid wirksamen Verbindungen untersucht, zur Kenntnis der für die corticoide Aktivität verantwortlichen Strukturen.

In letzter Zeit weckten die 16α-methylierten Corticosteroide und besonders das 16α-Methyl-$\Delta^{1,4}$-pregnadien-11β,17,21-triol-9α-fluor-3,20-dion (Dexamethason) ein grosses Interesse. Wir verdanken Oliveto einen wertvollen und relativ vollständigen Überblick über die Chemie dieser neuen Verbindungen[3]. Bekanntlich besitzt Dexamethason, im Gegensatz zum entsprechenden, den 16α-Methylrest nicht enthaltenden Sterol, eine viel niedrigere mineralcorticoide Aktivität, wobei die bei den natürlichen und manchen 9-fluorierten Corticosteroiden vorkommende ungünstige Natriumretention zum grössten Teil aufgehoben wird[4,5]. Somit spielt Dexamethason heute praktisch eine wichtige therapeutische Rolle bei rheumatischen Erkrankungen.

Die durch die 16α-Methylierung verursachte Hemmung des Natriumchlorid-retentions haben wir jetzt bei Verwendung eines typischen mineralcorticoid wirkenden Stoffes, des Desoxycorticosteron-acetats (DCA) untersucht. Die biologische Aktivität des 16α-Methyl-Δ^4-pregnen-21-ol-3,20-dion 21-acetats (16α-Methyl-DCA) wurde mit jenem des Desoxycorticosteronacetats durch die Bestimmung der Leberglykogenablage und der Elektrolytenausscheidung bei adrenalektomisierten Ratten verglichen. 16α-Methyl-DCA wurde nach der Methode von Ringold und Stork[6] aus 16α-Methylprogesteron (16α-Methyl-Δ^4-pregnen-3,20-dion) hergestellt. Letzteres wurde nach Marker und Crooks[7] aus $\Delta^{5,16}$-Pregnadien-3β-ol-20-on durch Einwirkung des Grignardreagenzes und anschliessende Oppenhauer-Oxydation des erhaltenen 16α-Methyl-Δ^5-pregnen-3β-ol-20-ons dargestellt. Die α-Stellung des Methylrestes am C-16 dieser Verbindungen haben Arth *et al.*[8] und Gallagher *et al.*[9] bewiesen. 16α-Methylprogesteron wird bei starker Belichtung, unter heftigem Rühren und bei Zimmertemperatur in Methanol-tetrahydrofuran-Lösung 6 h lang mit Jod und fein pulverisiertem Calciumoxyd behandelt. Die entfärbte Lösung wird in Eiswasser gegossen, mit Essigsäure angesäuert und das gebildete 21-Jodderivat mit CH_2Cl_2 extrahiert. Die eingeengten organischen Auszüge werden ohne jede weitere Reinigung mit geschmolzenem essigsaurem Kalium in Aceton 16 h zum Rückfluss erhitzt. Nach Einengen wird das 21-Methyl-DCA mit CH_2Cl_2 extrahiert und dreimal aus Essigester umkristallisiert. Smp. 139 bis 142°C; $[\alpha]_{20}^{D} = +136{,}5$ ($c = 1{,}029$ Dioxan). Analyse für $C_{24}H_{34}O_4$ (Mol.-Gew. 386,512). Gef. C% 74,5; H% 9,0. Ber. C% 74,58; H% 8,81. Das IR-Spektrum ist unten angegeben.

Für die biologischen Versuche wurden männliche Ratten, 5 Tage nach Adrenalektomie, mit mittlerem Körpergewicht von 140 g verwendet. Nach 15 h Hungern wurden die Tiere einzeln in Urin-Sammelkäfige gestellt und subkutan mit 0,2 cm³ einer Sesamöl-Lösung des Sterols und gleich nachher intraperitoneal mit 5 cm³ einer bei 37°C erwärmten physiologischen Lösung behandelt. Der Elektrolytengehalt (Na und K) des Urins wurde spektrophotometrisch (Bayrd-Flammenspektrophotometer) und das Leberglykogen bei mit Phenobarbital (Na-Salz) getöteten Tieren 8 h nach Verabreichung des Produktes

Tab. I. Wirkung des DCA und des 16α-Methyl-DCA auf Urinausscheidung und auf Natrium- und Kaliumgehalt im Urin von adrenalektomisierten Ratten.

	Behandlung µg/Ratte	Nr. der Tiere	Urin [a]	Na [b]	K [b]	Na/K	± Standard Fehler
DCA	100	10	0,21	0,39	0,75	0,52	± 0,09
DCA	250	10	0,19	0,20	0,69	0,34	± 0,04
16α-Methyl DCA	100	10	0,24	0,71	0,61	1,16	± 0,19
16α-Methyl DCA	250	10	0,18	0,44	0,47	0,94	± 0,10
Kontrolle		10	0,30	0,81	0,59	1,37	± 0,16

[a] cm³/h/100 g Tiergewicht [b] mg/h/100 g Tiergewicht

Tab. II. Glykogenablage in der Leber adrenalektomisierter Ratten.

	Behandlung µg/Ratte	Leberglykogen (mg/100 g Rattengewicht)	± Standard-Fehler
DCA	100	1,12	± 0,17
DCA	250	1,32	± 0,04
16α-Methyl-DCA	100	1,10	± 0,11
16α-Methyl-DCA	250	1,27	± 0,12
Kontrolle		1,00	± 0,10

nach Olson *et al.*[10] bestimmt. Die Verabreichung der Sterole erfolgte in Mengen von 100 γ und 250 γ, und mit jeder Dosierung wurden 10 Tiere behandelt, während die Kontrolltiere nur das Öl und die physiologische Lösung erhielten. Die Ergebnisse der Versuche sind in den Tabellen I und II zusammengestellt.

Aus den angegebenen Werten ist leicht zu erkennen, dass das 16α-Methyldesoxycorticosteronacetat auf die Elektrolytenausscheidung im Urin nur einen sehr geringen Einfluss hat, der auch nur mit den höheren Mengen (250 γ) signifikant wird. Im Glykogenablage-Test erweist sich das 16α-Methyl-DCA, genau wie das DCA selbst, als praktisch inaktiv.

Man sieht daraus, dass die Natriumretentionshemmung, die bei Einführung eines α-Methylrestes am Kohlenstoff 16 des DCA eintritt, in auffallender Weise derjenigen ähnlich ist, welche die gleiche Strukturänderung des 9α-Fluorprednisolons hervorruft: man kann deshalb wahrscheinlich von einer spezifischen Eigenschaft der α-Methylierung am C-16 sprechen. In der Tat ist im Falle des 16α-Methyl-DCA jede gegenseitige Wirkung der glycocorticoiden Aktivität ausgeschaltet: anderseits fehlt im

* Britische Patentanmeldung Nr. 39037/58 vom 3. 12. 1958.

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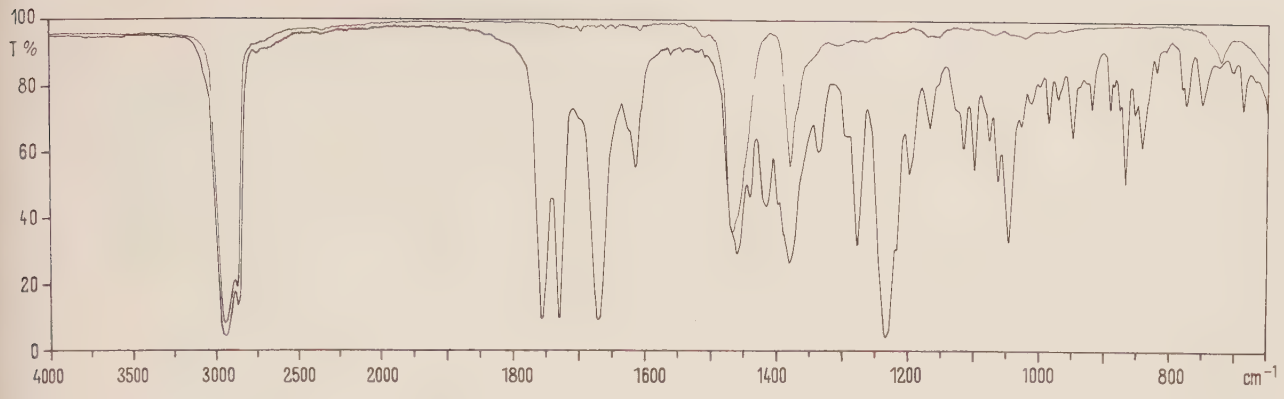

Infrarotspektrum des 16α-Methyl-DCA in Nujol

Vergleichsprodukt (DCA) ein 9α-Fluorrest, der die mineralcorticoide Aktivität anregen könnte, und gegen welche der 16α-Methylrest eine intramolekulare gegenseitige Wirkung ausüben könnte.

Da nun das 16α-Methyl-DCA keine nennenswerte glycocorticoide Wirkung auf die Leberglykogenablage bei der Ratte zeigt, glauben wir bewiesen zu haben, dass der 16α-Methylrest als solcher kein «Träger» der glycocorticoiden Aktivität beim Dexamethason, 16α-Methylprednisolon und weiteren 16α-Methyl-Sterolen sein kann.

Nachschrift: Nachdem die Arbeit zum Druck geschickt worden war, haben auch V. Petrow und D. M. Williamson, J. Chem. Soc. *1959*, 3595, über die Herstellung durch andere Verfahren des 16α-Methyl-desoxycorticosteron-acetats referiert.

G. Maffii, L. Fontanella,
P. Schiatti und E. Testa

Forschungslaboratorien der Lepetit S. p. A. Milano (Italien), 4. Dezember 1959.

Summary

The introduction of a 16α-methyl group in desoxycorticosterone acetate, a typical mineral-corticoid hormone, abolishes the sodium retention activity of desoxycorticosterone acetate. Since the same effect of 16α-methylation had been previously noted by other authors in 9α-fluoroprednisolone, our results further support the hypothesis that the 16α-methyl group is responsible for this specific change of metabolic activity in steroids.

Studies on Wound Healing: 1. Metabolism of ^{35}S in 'Repair Tissue' of Skin Wounds during the various Phases of Healing[1]

It is well known that 95% of ^{35}S parenterally injected in the form of $Na_2S^{35}O_4$ is rapidly eliminated through feces and urines (Dziewiatkowski[2]); the remaining 5% is distributed in the various organs according to a tropism which differs greatly from tissue to tissue (Dziewiatkowski[3], Campbell *et al.*[4], Boström[5], Jorpes *et al.*[6], Asboe-Hansen[7], Boström *et al.*[8]).

The maximal amount of ^{35}S stored by the organism is taken up by the bone tissue, bone marrow, and cartilage, chiefly in the form of chondroitinsulphuric acid (Dziewiatkowski *et al.*[9,10]).

Boström[11] demonstrated that the dermis is also capable of taking up ^{35}S, though in much smaller quantity than the above – mentioned tissues, incorporating it almost entirely into the chondroitinsulfuric acid of the ground substance.

It was observed by Layton[12] that embryonic tissues, especially granulation tissues, have a property of taking up much larger amounts of ^{35}S than the corresponding normal adult tissues.

He observed also that the uptake of ^{35}S is influenced by some hormones: thus cortisone, for instance, reduces considerably such ability.

The present investigations aim at studying the metabolism of ^{35}S in 'repair tissue' of skin wounds experimentally produced in laboratory animals.

Materials and Methods. 7 guinea pigs of both sexes weighing from 220 to 295 g were used in these experiments. Throughout the investigation, the animals were fed on a diet containing sufficient amounts of proteins and vitamins to cover the organism requirements.

After back-shaving and skin-disinfection, each guinea pig was inflicted a quadrangular wound of 2 cm side in the intrascapular region. The wounds, involving both skin and underlying subcutaneous tissue, were inflicted so that at a given moment the animals presented at the same time differently dated wounds, therefore different stages of repair tissue.

Two days before the animals were sacrificed, each guinea pig was intravenously injected with 5·678 μc of ^{35}S (in the form of $Na_2S^{35}O_4$ in aqueous solution)/1 g body weight. The wound-covering tissue, as well as a sheet of normal skin and subcutaneous tissue (taken as control), were excised, carefully weighed, then mineralized for 18 h with H_2SO_4 at 95–96% in 50 ml Kieldahl flasks. Each solution was then vaporized until a dry residue was obtained, which was diluted into 10 ml distilled boiling water and, after cooling, was put into a Geyger-Müller

[1] For 'repair tissue' we mean that tissue (first granulation, then scar tissue) which fills up and repairs any loss of substance in the soft tissues of the animal organism.

[2] D. D. Dziewiatkowski, J. biol. Chem. *178*, 197 (1949).

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counter for fluids (Twentieth-Century-Electronics, Type M. 6) in order to dosage the radiations emitted by the ^{35}S contained in the various tissue extracts.

Values obtained in the various estimations have been worked out so as to express the 'concentration index', that is the relationship between the relative specific activity (R. S. A.) of repair tissue and that of normal skin and subcutaneous tissue.

Results. Values obtained through the present experiments are reported in the Table and in the Figure.

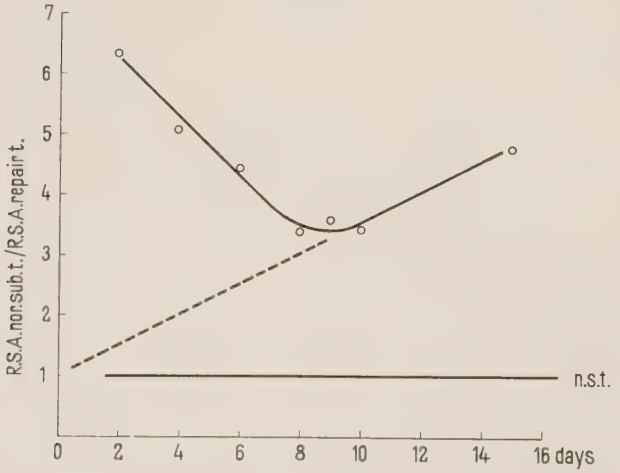
Discussion. The ability of repair tissue to take up a larger amount of ^{35}S that is done by normal skin and subcutaneous tissue (these data being in perfect agreement with those of Layton[13]) points a considerably increased metabolism of ^{35}S in the repair tissue.

The very high initial values might find a logical explanation by admitting that ^{35}S in repair tissue is not at first incorporated in the SO_4-group of chondroitinsulphuric acid, since the mechanism of its incorporation surely requires a comparatively long time, but bound to some substance or group of substances present in the tissue, which would take it up in considerable quantities and in a relatively labile way. It is probably only with time, probably in relation to the changes observed by several authors both in the fibrocellular part (Dumphy *et al.*[14], Sylvén[15] etc.) and in the ground substance (Sylvén[16], Campani *et al.*[17] etc.) of repair tissue, that ^{35}S pass from 'uptake' to 'incorporation'.

Relative specific activity (R. S. A.) of normal cutaneous and subcutaneous, and of repair tissue of skin wounds at various stages of the healing process.

Guinea pig No.	Tissue type	R. S. A. Normal cutaneous tissue	R. S. A. Repair tissue	Concentration index
1	2-days rep. t.	0·0432825	0·2732230	6·31
2	4-days rep. t.	0·0171934	0·0874926	5·08
3	6-days rep. t.	0·0202942	0·0903792	4·45
4	8-days rep. t.	0·0783679	0·2672030	3·40
5	9-days rep. t.	0·0163303	0·0582180	3·56
6	10-days rep. t.	0·0221658	0·0758071	3·42
7	15-days rep. t.	0·0164203	0·0778325	4·74

$$\text{R.S.A.} = \frac{\mu c \text{ observed}}{\text{sample weight}} \cdot \frac{\text{body weight}}{\mu c \text{ injected}} .$$



Behaviour of the ^{35}S content in the repair tissue of cutaneous wounds

The lowest values of ^{35}S found between the sixth and tenth day are perhaps the expression of the metabolism cycle of chondroitinsulphuric acid of repair tissue.

The amount of ^{35}S incorporated into the chondroitinsulphuric acid of wound repair tissue might probably be expressed as a straight line which, starting from very low values, joins the curve of the Figure by the eighthtenth day and, from that point on, follows it. The large amount of ^{35}S found in the early days might in fact be the result of the ^{35}S uptake plus incorporation, since the method used in these experiments does not allow such differentiation.

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Istituto di Patologia Chirurgica, Università di Pavia (Italy), December 8, 1959.

Riassunto

1) Il «tessuto di riparazione» delle ferite cutanee ha la proprietà di «fissare» una quantità di S^{35} notevolmente più grande di quella captata dal connettivo sottocutaneo.

2) La quantità di S^{35} fissata varia durante le diverse fasi del processo di guarigione delle ferite: è molto alta nei primi giorni; scende quindi sino al sesto-decimo giorno, epoca nella quale raggiunge i valori più bassi, e risale poi gradualmente sino al quindicesimo giorno (ultimo giorno preso in considerazione nelle presenti ricerche).

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[15] B. Sylvén, Acta chir. scand. *86*, Suppl. 66 (1941).
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Queen Recognition by Worker Honeybees (*Apis mellifera* L.)

Introduction. The sight, sound and scent of a queen honeybee do not inhibit either queen rearing by worker bees[1–3] or the development of their ovaries[4–10]. Inhibition occurs only when the bees can touch their queen and obtain 'queen substance' from her[3,10].

Many beekeepers believe that a queen has an odour attractive to workers. If this true it would be important because the odour would help workers to find the queen and get queen substance, but the only experimental evidence even suggestive of a queen odour seems to be that provided by Lecomte[11], Voogd[12], and Pain[13].

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[2] A. Müssbichler, Z. vergl. Physiol. *34*, 207 (1952).
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[4] A. P. de Groot and S. Voogd, Exper. *10*, 384 (1954).
[5] J. Pain, Insectes sociaux *1*, 59 (1954).
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[9] C. G. Butler, Exper. *13*, 256 (1957).
[10] C. Verheijen-Voogd (née S. Voogd), Z. vergl. Physiol. *41*, 527 (1959).
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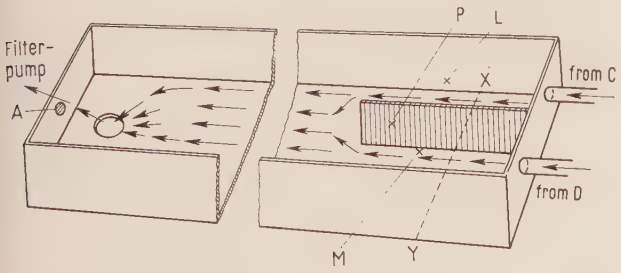
The following experiments attempted to find whether a mated queen honeybee has an odour that is attractive to workers and, if so, whether it is produced by her queen substance or something else.

Method. Experiments were made in red light to which workers do not respond, using an olfactometer (Fig. 1) designed by FREE and BUTLER[14,15]. Air was drawn through it as indicated. 'C' and 'D' each contained a different test material, and the air-flow through them was kept as constant and equal as possible.

One worker was tested at a time. It was introduced through 'A' and moved forward against the air stream until it reached the partition 'P' when it had a choice of chambers 'L' and 'M'. When it passed the line 'XY' the chamber concerned was recorded. The bee was then removed, the containers 'C' and 'D' interchanged and another bee put in. Twenty bees at least, taken at random from normal queenright colonies, were used in each experiment. The whole apparatus was washed between experiments and whenever a bee excreted in it. No bee was used more than once.



Olfactometer arrows indicate direction of air flow from the containers C and D whole apparatus made od 'Perspex'.

Materials tested. All the queens and workers used in containers 'C' and 'D' were dead. All the queens, except Nos. 141 and 2, had been dried for at least 4 months. The sample of pure crystalline queen substance was extracted and purified by Dr. R. K. CALLOW and Miss N. C. JOHNSTON of the National Institute for Medical Research, Mill Hill, London, using the method described by BUTLER, CALLOW, and JOHNSTON[16].

'Extracted queens' were queens from which queen substance was removed in ethanol in a micro-Soxhlet apparatus for 48 h.

Solutions in ethanol of pure queen substance, of the materials extracted from whole mated queens, and pure ethanol, were placed on filter-paper in the containers 'C' and 'D'.

Results and conclusions. The results, summarised in the Table, support the idea that queens, alive or dead, attract worker bees at a distance.

FREE and BUTLER[14] showed that even a large number of newly killed worker bees has no odour attractive to other workers; Experiment 1 supports this conclusion.

Of 6 mated queens tested individually (Experiments 2, 3, 4, 5, 9, 10) only one (No. 3) attracted individual workers towards it in preference to the odours of either dead workers, queens that had been extracted in ethanol, or nothing. Even a freshly killed queen (No. 141) was not attractive, but when two or more of these 6 queens were used together their combined odour was atractive (Experiments 6, 7, 8, 11). Therefore, queen honeybees not only produce an odour attractive to worker bees, but this

Results of olfactometer experiments

Exp. No.	No. bees tested	Material in 'C'	Material in 'D'	No. bees that moved towards 'C'	Significance
1	80	12 workers	nil	38	—
2	40	mated queen 1	nil	16	—
3	40	mated queen 141	nil	19	—
4	20	mated queen 2	1 worker	13	—
5	20	mated queen 3	1 worker	15	$P < 0{\cdot}05$
6	80	mated queens 1, 2	4 workers	52	$P < 0{\cdot}01$
7	40	mated queens 141, 1, 2	6 workers	34	$P < 0{\cdot}01$
8	80	mated queens 1–6	nil	63	$P < 0{\cdot}001$
9	20	mated queen 4	extracted mated queen 7	12	—
10	20	mated queen 5	extracted mated queen 8	13	—
11	20	mated queens 1–6	extracted mated queens 9–14	19	$P < 0{\cdot}001$
12	40	2 ml ethanol extract (A) mated queens (wet)	2 ml ethanol (wet)	27	$P < 0{\cdot}05$
13	60	2 ml ethanol extract(A) [a] mated queens (dry)	2 ml ethanol (dry)	35	—
14	40	2 ml ethanol extract(A) [b] mated queens (dry)	2 ml ethanol (dry)	21	—
15	20	2 ml ethanol extract(B) mated queens (wet)	2 ml ethanol (wet)	20	$P < 0{\cdot}001$
16	20	2 ml ethanol extract(B) [c] mated queens (dry)	2 ml ethanol (dry)	15	$P < 0{\cdot}05$
17	40	1·0 mg crystalline queen substance in 2 ml ethanol (wet)	2 ml ethanol (wet)	21	—
18	40	1·3 mg crystalline queen substance	nil	20	—

Extracts were placed on filter-paper and either used immediately before the solvent had evaporated (= wet) or after it had evaporated (= dry).
[a] = 2 h after Experiment 12; [b] = 17 h after Experiment 12; [c] = 1/2 h after Experiment 15.

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persists in dead queens dried for more than 4 months. The responsible substance is removed by extraction in ethanol (Experiment 11) and ethanol extracts also attract workers (Experiments 12, 15, 16).

Although the attractive odour persists for months on dried queens, it soon disappears when ethanol extracts are evaporated on filter-paper (Experiments 13, 14, 16). Presumably, therefore, the attractive odour is only gradually released from a queen's body, where it is perhaps being retained by her body waxes.

Queen substance can also be extracted from queens' bodies in ethanol[17] but pure queen substance does not have an odour that attracts workers even when it is offered in relatively large amounts – i.e. certainly several times the quantity present on the body surfaces of ten mated queens together[18] – (Experiments 17, 18) and on a filter-paper surface several times larger than the combined surface areas of 10 queens (Experiment 17). It is clear, therefore, that queen substance is not responsible for the attractive odour. The nature and origin of the responsible substance is unknown; its odour probably serves to attract workers to a queen, and so helps them to find queen substance, but the substance does not itself inhibit queen rearing[1–3].

C. G. Butler

Rothamsted Experimental Station, Harpenden (Hertfortshire, England), May 18, 1960.

Zusammenfassung

Begattete und sogar getrocknete Bienenköniginnen haben einen für Arbeiterinnen anziehenden Geruch, der jedoch, mit einer Ausnahme, nur bei Darbietung von mehr als einer Königin demonstriert werden konnte. Der anziehende Geruch ist wie «Queen Substances» mit Alkohol extrahierbar, stammt aber nicht von der «Queen Substance». Die Herkunft der riechenden Substanz ist nicht bekannt. Der Königinnengeruch dient wahrscheinlich zur Anlockung der Arbeiterinnen und damit zur Erleichterung des Auffindens der «Queen Substance», die allein die Königinnenaufzucht zu verhindern vermag, wenn sie in genügender Menge vorhanden ist.

Pressure Perception in *Ostariophysi*

Concerning the site of pressure perception in the *Ostariophysi*, there is much controversy in the literature. According to some authors, the Weberian ossicles and the labyrinth play a major role in the perception of changes of hydrostatic pressure[1–3]; according to others, pressure perception is mainly localized in the swimbladder wall[4–8], where abundant nerve endings have been described histologically[9–13], or in the surrounding tissues. It seems likely that both principles are realized, each of them under its own circumstances[14]. The following observations add new evidence to this problem.

Among a number of blinded minnows (*Phoxinus laevis*) from which both mallei were removed, some were precisely equilibrated hydrostatically. These fishes stayed almost motionless in the middle of the water, thus offering good opportunity to observe even slight reactions to pressure change. For some fishes, pressure increase was used as a stimulus, while pressure decrease was applied to others. In both cases, spontaneous reactions occurred on pressure changes of only a few (4–7) cm of water pressure (rate of pressure change: about 70 cm/min). At each pressure increase, the fish showed a sudden, quiet ascend (downward beating of pectoral fins) over several cm. This response was always preceded by an interval of 5–8 sec without any visible signs of pressure perception or passive displacement. Frequently, the active ascent occurred without any observable rotation of the animal around a transverse axis; the fish body remained quite horizontal. At each pressure decrease, a similar reaction in the opposite direction was observed. These observations show that a blind minnow without functioning Weberian apparatus is still sensitive to pressure changes of a few cm of water pressure, and that the Weberian ossicles play no main role in the maintenance of hydrostatic equilibrium.

It has been reported that blinded minnows can easily be trained to react to slight pressure changes, both pressure increase and pressure decrease (rate of pressure change about 120 cm water/min[15]). Determination of a threshold has not been tried, but a pressure change of $^1/_2$–1 cm of water pressure evoked still a clear response (feeding reaction). After removal of both mallei, these reactions were abolished, even when the pressure increase used as a training stimulus was put up to 40 cm. When repeating these training experiments, we found in 7 blind fishes without mallei, after a long series of trials (at least 40 stimulus-punishment or stimulus-food combinations) positive conditioning (escape or feeding reactions, respectively) to a pressure increase or decrease of only 7–8 cm water pressure (total number of combinations: 1100). With a pressure change of 5–6 cm, the percentage of positive reactions went down to 50%, and this value is evidently close to the threshold, especially in the case of pressure decrease. The results of our training experiments, therefore, agree well with the conclusion drawn from our earlier equilibrium experiments.

Summarizing, we may conclude that in *Phoxinus laevis* the Weberian apparatus and the labyrinth play no main part in pressure perception underlying the maintenance of hydrostatic equilibrium.

Z. Qutob

Laboratory of comparative Physiology, University of Utrecht (Holland), June 17, 1960.

Zusammenfassung

Blinde, malleuslose Elritzen beantworten unter günstigen Umständen schon Schwankungen von 4–7 cm Wasserdruck mit spontanen Reflexbewegungen. Druckzunahme löst – nach bestimmter Latenzzeit – Aufwärtsbewegung aus, Druckabnahme Abwärtsbewegung. Auch in langfristigen Dressurversuchen wurde entsprechende Reaktion auf Schwankungen bis zu 5–6 cm Wasserdruck erzielt.

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Reduced Sensitivity to some Drugs 48 h after Chlorpromazine Treatment

Previous experiments demonstrated that, in rats pretreated with electroshock, the sleeping-time induced by barbiturates (48–72 h later) is much longer than in normal animals[1].

It was supposed that this effect was not specific for electroshock treatment, but that also a pretreatment with certain substances could modify the effect of other drugs administered some time later.

Using chlorpromazine, the opposite effect to that shown after electroshock was observed. A single dose of chlorpromazine was able to reduce the sleeping-time when the barbiturates are given 48 h later.

This unexpected result was studied under different conditions. The following points are particularly stressed in the present research: (1) time between chlorpromazine administration and the onset of shortened barbiturate sleeping-time; (2) quantitative relation between the doses of chlorpromazine and the effects obtained; (3) possible different effects between single and repeated doses; (4) if this effect was specific for barbiturates or reproducible using other hypnotic agents; (5) possible hormonal interference; (6) a possible relation between the shortening of the sleeping-time and a faster breakdown of the hypnotic drugs.

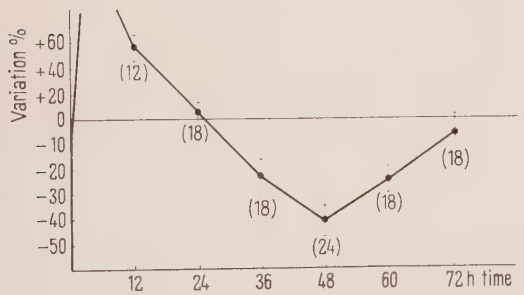
Sprague-Dawley female rats, weighing 200 g were used. Chlorpromazine was administered intraperitoneally at the dose of 15 mg/kg. Room temperature was 18–22° C.

The Figure shows the duration of sleep when pentobarbital was given at different intervals after chlorpromazine administration.

The synergistic effect of chlopromazine, when administered with barbiturates, is well known. This synergism is still noticeable after 12 h, but after 24 h has completely disappeared, until 72 h after the chlorpromazine administration, a markedly decreased sleeping-time is observed which reached a peak after 48 h. All further experiments were carried out using this time.

Our results on the relation between the dose of chlorpromazine used and the sleeping-time reduction are reported in Table I. In some experiments, chlorpromazine was administered for 5 days, before an interruption of 48 h.

The sleeping-time decreases with increasing doses of chlorpromazine. When chlorpromazine is given for 5 successive days and the treatment is then discontinued, as usual, for 48 h; the shortening of the sleeping-time is even more remarkable.



Variation of pentobarbital induced sleeping-time after a single injection of chlorpromazine
The groups after 36 and 60 h are significantly different (versus controls) at the level of $p < 0{\cdot}01$

In Table II results are collected concerning the animals pretreated with chlorpromazine under the usual experimental conditions and injected after a 48 h interval, with 'Doriden', 'Viadril', ethyl alcohol, and hexobarbital.

The effect observed with pentobarbital is also evident in the case of the hexobarbital. Neither with 'Doriden' nor 'Viadril' and ethyl alcohol there was any noticeable variation of the sleeping-time observed. It may be deduced that the chlorpromazine pretreatment does not induce an aspecific resistance against narcotic drugs. There is a specific effect against pentobarbital and hexobarbital, consistent with the hypothesis that the chlorpromazine pretreatment is able to modify the metabolic pathways of barbiturates.

Tab. I. Effect of different doses of chlorpromazine pretreatment on the sleeping time of pentobarbital.

Dose (i. p.)	No. of animals	Sleeping-time (min) ± S.E.	% variation	p
I Controls	18	84 ± 3·9	—	—
2·5 mg/kg 1 day	8	78 ± 4·4	− 7	n. s.
5 mg/kg 1 day	18	64 ± 2·4	− 24	$p < 0{\cdot}001$
10 mg/kg 1 day	18	53 ± 3·8	− 37	$p < 0{\cdot}001$
15 mg/kg 1 day	18	49 ± 4·5	− 42	$p < 0{\cdot}001$
II Controls	14	74 ± 5·7		$0{\cdot}02 >$
2·5 mg/kg 5 days	14	57 ± 3·4	− 23	$p > 0{\cdot}01$
5 mg/kg 5 days	13	46 ± 4·0	− 37	$p < 0{\cdot}001$
10 mg/kg 5 days	14	39 ± 4·4	− 46	$p < 0{\cdot}001$

25 mg/kg pentobarbital were injected i. p. 48 h after the last injection of chlorpromazine.

Tab. III. Influence of pretreatment with chlorpromazine on 'Doriden', 'Viadril', ethylalcohol, hexobarbital narcosis.

Pretreatment	Narcosis	No. of animals	Sleeping-time (min) ± S.E.	p
Chlorpromazine	Doriden	7	80 ± 8·7	n. s.
	Doriden	6	76 ± 6·5	
Chlorpromazine	Viadril	8	49 ± 9·6	n. s.
	Viadril	8	66 ± 9·6	
Chlorpromazine	Alcohol	7	31 ± 4·8	n. s.
	Alcohol	7	46 ± 5·9	
Chlorpromazine	Hexobarbital	15	69 ± 3·2	$0{\cdot}01 >$
	Hexobarbital	16	53 ± 4·5	$p > 0{\cdot}001$

'Doriden' (85 mg/kg), 'Viadril' (70 mg/kg), 'ethyl alcohol' (40%, 10 cm³/kg), and 'hexobarbital' (95 mg/kg) were injected intraperitoneally.

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[7] Research fellow from Keio University, Tokyo (Japan).

Tab. III. Influence of pretreatment with chlorpromazine on pentobarbital narcose in adrenalectomized[a] or immature[b] rats.

Treatment	No. of animals	Sleeping-time (min) ± S.E.	% variation	p
I Controls	16	18 ± 3·9	—	—
Adrenalectomized rats	14	35 ± 5·0	—	—
Adrenalectomized rats + Chlorpromazine	12	11 ± 3·2	−68	$0{\cdot}01 > p > 0{\cdot}001$
II Immature rats	14	41 ± 2·9	—	—
Immature rats + Chlorpromazine	14	26 ± 2.9	−37	$p < 0.001$

[a] Female rats weighing 180 g, adrenalectomized 7 days before narcose and treated daily with 1 mg/kg cortisone acetate (s. c.). All rats were kept at 25°C room temperature and received 1% NaCl solution, as drinking water. Chlorpromazine was injected at the dose of 15 mg/kg and pentobarbital at the dose of 20 mg/kg i. p.

[b] Female immature rats (30 days old, average body weight 100 g) are used. The treatment dose of chlorpromazine was 10 mg/kg (i. p.) pentobarbital (22 mg/kg) was injected i. p.

It is well known that the barbiturate breakdown is modified by the cortical[2] and sex[3] hormones. For this reason we studied the late effect of chlorpromazine in adrenalectomized and immature rats. Our results are reported in Table III.

From our data it is possible to conclude that chlorpromazine pretreatment is able to reduce pentobarbital sleeping-time after 48 h also in the adrenalectomized and immature rats.

A possible decrease of barbiturate levels in brain, when the sleeping-time was reduced, was also investigated. In experiments injecting 15 mg/kg of chlorpromazine, and 48 h later, 25 mg/kg of pentobarbital, it was observed that the barbiturate concentration in brain of animals killed 1 h after the administration, was only 60% in comparison with that of the controls[4].

Our results can be explained with a decreased penetration of barbiturates into the brain or with an enhanced breakdown. The second effect is more probable. Recently, Remmer[5] observed an increase of hexobarbital oxidation by the liver microsome enzymes in rats pretreated with barbiturates. On the other hand, in very recent experiments, we were able to observe that chlorpromazine pretreatment can decrease the toxicity of strychnine and picrotoxin and the pharmacological effects of meprobamate and myanesin[6].

R. Kato[7]

Istituto di Farmalogia della Università di Milano (Italy), December 20, 1959.

Riassunto

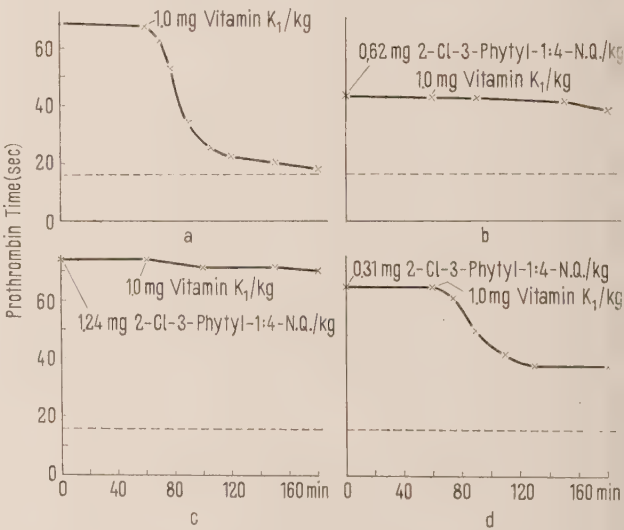
L'autore osserva che un trattamento fatto 48 h prima con cloropromazina induce nei ratti una diminuzione nel tempo di sonno da pentobarbital e da esobarbital, che si accompagna a una diminuzione della concentrazione encefalica dei barbiturici. Il fenomeno non si avvera per il sonno da «Doriden», nè da «Viadril», nè da alcool.

The Inhibition of the Antidotal Activity of Vitamin K_1 against Coumarin Anticoagulant Drugs by its Chloro Analogue[1]

The replacement of a methyl group by a chlorine atom in a biologically active compound may result in an analogue with inhibitory activity. The application of this principle has led to the synthesis of chlorine analogues of riboflavine which are competitive inhibitors[2,3]. Since vitamin K_1, 2-methyl-3-phytyl-1:3-naphthoquinone, contains a methyl group, its chloro analogue, 2-chloro-3-phytyl-1:4-naphthoquinone, has been synthesized and tested for its ability to inhibit the antidotal activity of vitamin K_1 against coumarin anticoagulant drugs.

Rabbits weighing from 2·5 to 3·5 kg were fed Warfarin [3-(α-phenyl-β-acetylethyl)-4-hydroxycoumarin] using a dose of 15 mg/kg of body weight/day for two days. This increased the prothrombin time to approximately 70 sec compared to a value of 15 to 17 sec before treatment. In such animals, the intravenous administration of 1 mg/kg of body weight of vitamin K_1 resulted in a significant reduction of the prothrombin time within 40 to 60 min (Fig. a). When the chloro analogue was administered intravenously 60 min before, 1·24 mg and 0·62 mg/kg completely blocked (Fig. b and c), whereas 0·31 mg/kg significantly reduced the antidotal effect of vitamin K_1 (Fig. d).

The precise site or mode of action of vitamin K is not known. Quick[4] and Almquist[5] have suggested that certain plasma clotting factors (prothrombin, factors VII, IX, X) are synthesized by an enzyme system of which the vitamin is a coenzyme. Because all compounds with Vitamin K activity are para-quinones, they may function as components of an electron transport system. Coumarin anticoagulaut drugs must interfere with this system by depleting it of the coenzyme, as their effect is reversed readily



a) Vitamin K_1 alone, b) c) d) 1·24 mg, 0·62, 0·31 mg respectively o 2-Chloro-3-Phythl-1:4-Naphthoquinone 60 min before the administration of Vitamin K_1.

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by vitamin K. The 2-methyl group also is essential for vitamin K-like activity and perhaps serves to anchor the vitamin K molecule to the apoenzyme[6]. While the methyl group and the chlorine atom have similar shapes, they differ in their polar resonance and inductive effects. The replacement of the 2-methyl group by a chlorine atom can result in an analogue which is sufficiently similar to occupy the free receptor sites on the surface of the apoenzyme in the coumarin anticoagulant treated animal but which is unable to elicit the biological effects of vitamin K.

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Department of Physiology and Pharmacology, University of Saskatchewan, Saskatoon (Canada), January 4, 1960.

Zusammenfassung

Die Wirkung von Vitamin K_1 auf mit Coumarin behandelte Kaninchen wird durch 2-Chloro-3-Phytyl-1:4-Naphthochinon gehemmt.

Uptake of ^{35}S Labelled Sulfate in the Exorbital Lacrymal Glands of Adult and Newborn Rats under Different Hormonal Treatment

Previous experiments have shown that testosterone treatment in normal and castrated male rats results in morphological changes of the exorbital lacrymal glands (Loewenthal glands), i. e. appearance of a tubular structure with mucous secretion and increase of the nuclear volumes (Cavallero and Morera[1]). An additional research has been now undertaken in order to show whether or not testosterone, in comparison with other hormones, is capable of affecting the uptake of ^{35}S labelled sulfate by the glands both in adult and newborn rats.

In a first experimental series, adult male castrated rats were used. Four animals without hormonal treatment and four treated with testosterone propionate (1 mg daily subcutaneously for 10 days) were injected intraperitoneally with ^{35}S labelled Na_2SO_4, at a dosage of 0·3 μC/g body weight. 18 h after the injection they were sacrificed, their exorbital lacrymal glands were carefully dissected, weighed, and dissolved in 0·5 cm^3 of conc. hydrochloric acid. The solution was then dried with infrared rays and the radioactivity of the dry material evaluated with a Geiger counter (mica-end-window weighing 2·6 mg/cm^2). The radioactivity was expressed as counts/min/mg wet weight.

From this first experiment, it was found that the ^{35}S-uptake was higher for testosterone-treated glands; the mean count/min/mg was 11 for the control glands, whereas 27 impulses were counted with the testosterone-treated glands, the difference being highly significant.

In a second experimental set, two-days old rats were used, partly untreated and partly treated with various hormones, as reported in the Table. Hormonal treatment lasted three days; thereafter radiosulphate, 1 μC/g of body weight, was injected. Other procedures were identical to those of the first series. The data were analyzed statistically by the analysis of variance technique. Statistical significances were assessed at 1% level.

^{35}S uptake in the exorbital lacrymal glands of 2-days old rats under different hormonal treatment.

No. animals	Treatment	Daily dose	Net counts/ min/mg
8	Controls	—	33
5	Testosterone	0,1 mg	36
5	TTH (Organon)	0·2 mg	43[a]
6	Thyroxine (Roche)	0·01 mg	37
5	Cortisone	0·1 mg	17[a]
4	Insulin	0·1 I. U.	30
4	Glucagon (Lilly)	0·1 mg	20[a]

[a] Differs significantly from control group.

From this Table, it appears that in newborn rats no change occurs in the ^{35}S uptake by the glands after testosterone, thyroxin, and insulin treatment; on the other hand, there is an increase following thyrotropin and a decrease after both cortisone and glucagon treatment. Our results with thyrotropin are similar to those obtained by Wegelius *et al.*[2] on other lacrymal glands (ventral and Harderian glands) of the guinea pig, where hormonal treatment actually increased ^{35}S uptake.

Histological studies on adult Loewenthal glands have shown that testoterone treatment results in important morphological changes; i. e. appearance of a tubular structure with collection of mucous material in the lumina, associated with increased ^{35}S uptake. On the other hand, histological examination of the Loewenthal gland in newborn rats showed evidence of a conspicuous structural immaturity; in addition, the treatment with testosterone did not elicit the structural modifications seen in the adult, nor cause any change in the uptake of ^{35}S.

We can thus conclude that Loewenthal glands of the newborn animal are not sensitive to testosterone action, while being quite responsive to the action of TTH, or, more precisely, to the ophthalmotropic activity of the complex thyrotropic hormone, behaving like the other lacrymal glands. Moreover, there seems to be a close relationship between mucous changes of the glands and increased ^{35}S uptake.

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Department of Pathological Anatomy, University of Pavia, Radioisotope Unit, Hospital of Busto Arsizio and Institute for Stomatology, Milano (Italy), January 23, 1960.

Zusammenfassung

Die Verfasser weisen nach, dass die Testosteronbehandlung in der äusseren Orbitaldrüse (Nebenohrspeicheldrüse; Loewenthalsche Drüse) der erwachsenen Ratte die Aufnahme von ^{35}S steigert. Gleichzeitig wird eine tubuläre Veränderung der Drüse mit schleimiger Sekretion beobachtet. Diese Erscheinungen sind beim neugeborenen Tier nach Testosteron nicht nachweisbar, hingegen wird hier durch Thyrotropin die ^{35}S-Aufnahme gesteigert.

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Effects of Potassium Ions on Brain Respiration and Aminoacid Incorporation into Brain Proteins *in vitro*

The stimulating effects of potassium ions on brain respiration were shown many years ago by Ashford and Dixon[1] and Dickens and Greville[2]. This stimulation, which seems greatly to resemble that brought about by oscillating electric pulses, is highly sensitive to drugs that have no demonstrable effect on the unstimulated respiration in the presence of glucose[3].

In the course of researches on the metabolism of brain cortex slices from hypoxic rats, preliminary experiments have been carried out, in an attempt to gain a better understanding of the relationship between stimulation of brain respiration by potassium ions and incorporation of 1-^{14}C-glycine into the proteins of brain cortex slices incubated *in vitro*.

All the experiments have been carried out on brain cortex slices from albino rats of either sex, weighing about 200 g. The slices were incubated in a conventional Warburg apparatus, at 37°C, the gas phase being air in some experiments and oxygen in others. Different phosphate salines, having respectively the following compositions, were used: (A) $1{\cdot}28\times10^{-1}$ *M* NaCl; $1{\cdot}35\times10^{-2}$ *M* KCl; $1{\cdot}94\times10^{-3}$ *M* $CaCl_2$; $6{\cdot}8\times10^{-4}$ *M* $MgSO_4$; 1×10^{-2} *M* sodium phosphate buffer, pH 7·2; 2×10^{-2} *M* glucose. (B) $2{\cdot}0\times10^{-2}$ *M* NaCl; $1{\cdot}2\times10^{-1}$ *M* KCl; the other components as in (A). (C) $1{\cdot}28\times10^{-1}$ *M* NaCl; $1{\cdot}2\times10^{-1}$ *M* KCl; the other components as in (A). Phosphate salines A and B had a final concentration of 0·319 os*M*/l; phosphate saline C had a final concentration of 0·531 os*M*/l.

The incorporation of 1-^{14}C-glycine into the proteins of brain cortex slices was studied as described in previous papers[4,5], using phosphate salines A and B as incubation mediums.

From the data given in Table I, it can be seen that the stimulation of brain respiration induced by potassium ions in the presence of glucose takes place only if the slices are incubated in oxygen ($P < 0{\cdot}01$), and not if they are incubated in air ($P > 0{\cdot}05$). When the slices are incubated in oxygen, the respiration is significantly increased if the slices are incubated in the presence of large quantities of potassium, even if large amounts of sodium are present in the medium, which is therefore hypertonic ($P < 0{\cdot}01$). If large quantities of potassium are present in the medium, it is not possible to see any difference due to a different sodium concentration in the phosphate saline ($P > 0{\cdot}05$).

The results of the experiments on the incorporation of 1-^{14}C-glycine into brain proteins are given in Table II. It can be seen that the incubation of brain cortex slices in a potassium-rich phosphate saline, i. e. in a medium which induces an increase in the respiration of the slices, does not induce a similar increase in amino acid incorporation. On the contrary, this is markedly inhibited.

Our observations are in keeping with the results obtained by several workers[6–8] with different biological materials, which however are not responsive to the respiratory stimulation by potassium ions. It had been reported by Rossiter[9] that an increased concentration of potassium ions provokes an increase of oxygen uptake by cat brain sections, and a diminution of the incorporation of ^{32}P into organic phosphorus components, as represented by lipid phosphorus, ribonucleic acid, and phosphoprotein. It must therefore be kept in mind that the stimulation of the respiration of brain cortex slices induced by potassium ions can be uncoupled from energy-requiring reactions, such as phosphorylation in the experiments of Rossiter, or glycine incorporation into proteins, as demonstrated in the present paper.

Tab. I. Oxygen consumption by rat brain cortex slices, suspended in different phosphate salines in the presence of 2×10^{-2} *M* glucose. Results expressed as Q_{O_2}. Means of 6 experiments ± s. e. m.

Phosphate saline	A	B	C
Gas phase: air	8·2 ± 0·31	8·1 ± 0·47	8·3 ± 0·38
Gas phase: oxygen	13·8 ± 0·60	16·4 ± 0·32	16·8 ± 0·36

Tab. II. Incorporation of 1-^{14}C-glycine into the proteins of rat brain cortex slices, incubated in two different phosphate salines, for 1 h, in the presence of 2×10^{-2} *M* glucose. Gas phase, oxygen. Means of 6 experiments ± s. e. m.

Phosphate saline	A	B
c. p. m./mg protein	65·4 ± 2·5	20·8 ± 2·1

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Maria Bassi and A. Bernelli-Zazzera

Istituto di Patologia generale, Università di Milano (Italy), February 11, 1960.

Riassunto

È stata studiata l'azione degli ioni potassio sulla respirazione di sezioni di corteccia cerebrale di ratto e sull'incorporazione di glicina-1-C^{14} nelle proteine di tali sezioni. Il potassio, che stimola la respirazione delle sezioni se queste sono incubate in ossigeno in presenza di glucosio, inibisce invece l'incorporazione di glicina-1-C^{14} nelle proteine delle sezioni stesse.

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PRO EXPERIMENTIS

A New Technique for the Fluorescent Labelling of Proteins

Coons'[1] technique of labelling antibody with fluorescein has proved to be a useful research tool in immunochemistry, virology, and bacteriology and has become a

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valuable laboratory aid in the rapid identification of pathogenic and non-pathogenic microorganisms[2]. Major obstacles to a more widespread use of this procedure have been the laborious preparation of the labelling dye and its instability, the problem of protein denaturation due to organic solvents used in the labelling reaction and insufficient labelling of the protein. These difficulties have largely been overcome by the use of more stable fluorescent labelling agents such as fluoresceinisothiocyanate[3], the modified method of conjugation with the aid of filter paper[4] or the use of the solid labelling agents without organic solvents[5]. However, none of these techniques is entirely free of disadvantages. Thus, some fluorescent dyes are not readily prepared in solid form; others may be too insoluble in organic solvents for convenient preparation of filterpaper strips containing the desirable concentration of dye or they may be too reactive to remain stable on cellulosic materials for prolonged periods of time.

We believe to have found an alternative method which affords great convenience in preparing a variety of fluorescent labelling materials and incorporates all the advantages of the above mentioned modifications of COONS' original technique. The procedure used in our laboratories consists of adsorption of the labelling dye onto specially dried diatomaceous earth (Celite, hyflo super-cel, Johns-Manville Co.) or the like and is reported below in detail. Such 'fluorescent Celite powders' are more conveniently prepared and stored in quantity than impregnated filter paper and are easier to dispense than the latter or the minute quantities of solid dye required for fluorescent labelling. Their tremendously large reactive surface permits drastic reduction of the reaction time; 30 min or less have been found adequate for satisfactory labelling of protein solutions compared with 12 to 24 h used in the older techniques.

Materials and Methods. The following three fluorescent dyes were investigated: (1) 5-dimethylaminonaphthalenesulfonylchloride[6], (2) Lissamine Rhodamine B 200 (sulfonylchloride)[6], and (3) fluoresceinisothiocyanate[6]. The preparation of our labelling agents is illustrated in the following example. 2 g of Lissamine Rhodamine B 200 (red fluorescence) were suspended in a solution of 4 g phosphorus pentachloride in 40 ml ethyleneglycol dimethylether (dried over calcium hydride) and heated under reflux for 30 min. The solvent and phosphorus oxychloride were then removed in a rotary evaporator under reduced pressure, the excess phosphorus pentachloride extracted three times with carbontetrachloride and the Rhodamine B 200-chloride dissolved in 150 ml dry chloroform. After filtration from insoluble material the chloroform solution was added to 15 g of Celite (hyflo super-cel) which had been dried by heating at 300° C for 10 min. The chloroform was then distilled under reduced pressure in a rotary evaporator and traces of solvent removed from the residual powder with a high vacuum pump. Such preparations, when stored at room temperature in a tightly closed container retained satisfactory labelling activity for at least one year (longest period tested). The amount of active labelling dye on the Celite was assayed colorimetrically by extraction with specially dried chloroform and alkaline hydrolysis of the extract. The readings obtained with the hydrolysate were compared with a standard curve prepared with Lissamine Rhodamine B 200 (sodium salt). Similar fluorescent labelling powders were prepared in this way from 5-dimethylaminonapthtalenesulfonylchloride and fluoresceinisothiocyanate.

For the labelling tests 10–40 mg of 'fluorescent Celite powder' was added to 3 ml of rabbit serum and 3 ml of 0·05 *M* sodium carbonate-bicarbonate buffer (pH 9) in a centrifuge tube. The mixture was shaken mechanically for 30 min and centrifuged. The supernatant was dialysed for three days against running water and subjected to paper-strip electrophoresis. Satisfactory labelling of the proteins was observed in each case.

Acknowledgements. I wish to express my thanks to Miss TIIU REBANE for technical assistance, Mr. M. BALLAS for the assays of the fluorescent dyes, Mr. J. FONTANA for the electrophoreses determinations, and Dr. J. GARVEY of the Calif. Institute of Technology for a gift of Rabbit serum.

H. RINDERKNECHT

California Corporation for Biochemical Research, Los Angeles (Calif.), February 1, 1960.

Zusammenfassung

Es wird eine neue Arbeitsweise zur Fluoreszenzmarkierung von Proteinen beschrieben unter Verwendung auf Celite adsorbierter Fluoreszenzfarbstoffe. Die neue Methode bietet gegenüber andern heute gebräuchlichen Verfahren bedeutende Vorteile.

[2] For review of the pertinent literature see H. UEHLEKE, Z. Naturforsch. *13b*, 722 (1958).

[3] J. L. RIGGS, Masters Thesis, Univ. Kansas (1957).

[4] M. GOLDMAN and R. K. CARVER, J. exp. Med. *105*, 549 (1957).

[5] J. D. MARSHALL, W. C. EVELAND, and C. W. SMITH, Proc. Soc. exp. Biol. Med., N. Y. *98*, 898 (1958).

[6] Available from California Corporation for Biochemical Research, Los Angeles (California).

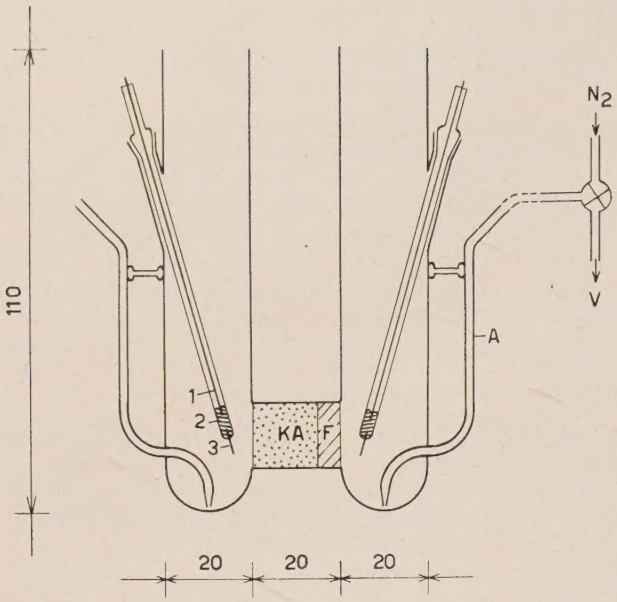
PRO LABORATORIO

Eine einfache Apparatur zur serienweisen Redoxtitration

Für redoxpotentiometrische Titrationen von säulenchromatographisch aufgetrennten Stoffen (Polyphenole, Askorbinsäure, Redoxfarbstoffe) ist eine Schnellmethode vorteilhaft, da meist mit einer grösseren Anzahl von Fraktionen gerechnet werden muss. Die nachstehend beschriebene Apparatur erlaubt in kurzer Zeit eine grosse Anzahl von Fraktionen zu titrieren.

Im linken Tubus befindet sich das Vergleichshalbelement (zum Beispiel Chinhydron) mit einer Platinelektrode. Es steht über eine KCl-Agar-Brücke (KA) und eine Glasfritte (F) mit dem Titrationsgefäss in Verbindung, in das eine zweite Elektrode eintaucht. Diese wurden nach dem von BIRNSTIEL[1] angegebenen Verfahren hergestellt. Quecksilber (2) gewährleistet die Verbindung zwischen dem Kupferdraht (1) und dem ins Glasrohr eingeschmolzenen Platindraht (3) vom Durchmesser 1 mm. Diese Elektroden münden schräg von der Seite her ein, damit die obere Tubusöffnung zum Einbringen der zu titrierenden Fraktion und der Titerlösung frei bleibt. Zur Gewährleistung steter Mischung während der Titration wird durch den Stutzen A ständig reinster Stickstoff zugeführt. Nach erfolgter Titration kann die Zelle mittels Wasserstrahlvakuum (V) ebenfalls über den Stutzen A geleert und gespült werden, wonach sie zur Aufnahme der nächsten Fraktion bereit ist. Auf die gleiche Weise kann auch der linke Tubus gereinigt werden. Die Grösse des Titrationsgefässes ist für Fraktionen von 7–20 ml berechnet.

[1] M. L. BIRNSTIEL, Diss. ETH. Zürich 1960.



H. V. Lott

Institut für Allgemeine Botanik, Eidgenössische Technische Hochschule, Zürich, 8. Mai 1960.

Summary

The use of the described apparatus for redoxpotentiometric titrations enables very rapid estimations with little manual effort.

ACTA COMPARATA

Fortschritte der Arzneimittelforschung[1]

Dem organischen Chemiker, befasse er sich mit der Isolierung, Konstitutionsaufklärung, Total- oder Partialsynthese eines Naturstoffes oder mit der synthetischen Gewinnung eines Heilmittels, stehen heute eine Reihe chemischer, physikalischer und analytischer Methoden zur Verfügung, die ihm erlauben, die gestellten Probleme, soweit sie eindeutig definierbar sind, meist innert relativ kurzer Zeit zu lösen. Für die Testierung und Evaluierung der hergestellten Präparate durch den Biologen und Mediziner sind ebenfalls moderne Methoden entwickelt worden, die wesentlich dazu beitragen, die pharmakologische Beurteilung eines Präparates in qualitativer und quantitativer Hinsicht zu beschleunigen. Es ist deshalb nicht verwunderlich, dass sich die Arzneimittelforschung in den letzten Jahrzehnten, insbesondere aber in den letzten 15 Jahren, geradezu stürmisch entwickelt hat; es ist weiter voraussehbar, dass diese Entwicklung mit noch gesteigerter Geschwindigkeit fortschreiten wird, wobei auch Gebiete, wie die Antivirus- und Antitumor-wirksamen Substanzen, wo noch keine praktisch befriedigende Lösung gefunden ist, einer solchen entgegengeführt werden.

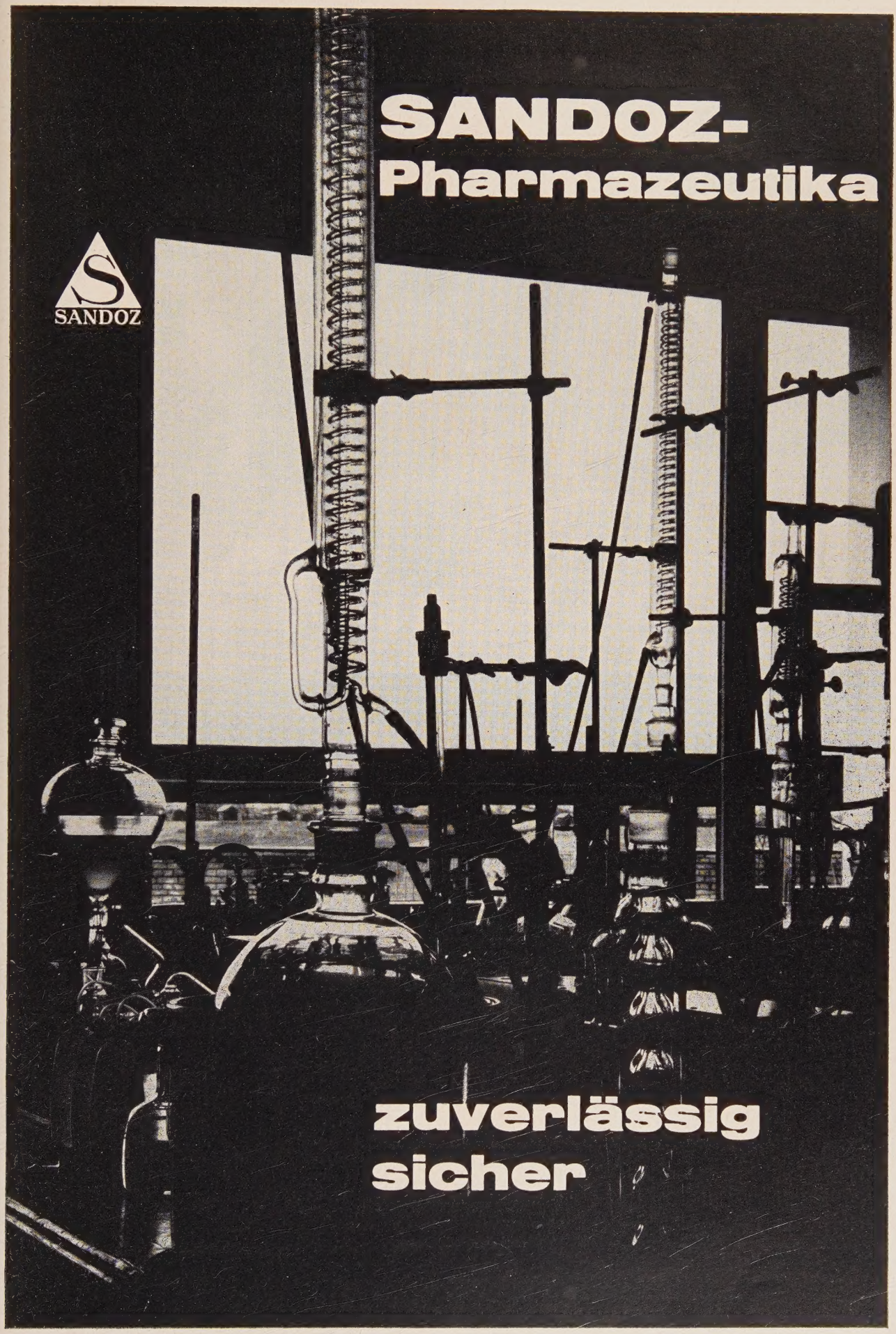
Für den Spezialisten, sei er Chemiker, Biologe oder Mediziner, wird es dabei immer schwieriger, sich einen Überblick über die eigenen und insbesondere die von ihm nicht direkt bearbeiteten Forschungszweige zu verschaffen. Zwar existieren spezialisierte Zeitschriften mit Kurzmitteilungen und gelegentlich Übersichtsreferaten und natürlich auch umfangreiche Monographien in Buchform über Spezialgebiete. Die meisten Informationen müssen aber aus einer Vielzahl von Zeitschriften mit im wesentlichen völlig anderer Hauptrichtung ausgesondert werden. Diese Tatsache hat zur Herausgabe der *Fortschritte der Arzneimittelforschung* geführt, in denen in zusammenfassenden Referaten periodisch über aktuelle Themen der Arzneimittelforschung berichtet werden soll. Natürlich kann und will eine solche Veröffentlichung das Studium der Originalliteratur nicht ersetzen; sie wird dem Forscher aber sicher den Zugang zu einem neuen Arbeitsgebiet wesentlich erleichtern oder ihn auf Arbeiten hinweisen, die er vielleicht übersehen hat.

Im ersten Band der genannten neuen Reihe, der sich in gediegener Aufmachung präsentiert, sind zuerst Beiträge über 5 Spezialgebiete enthalten. J. Büchi referiert über die chemischen Grundlagen der Jonenaustauscher und ihre praktische Anwendung bei der Herstellung von Heilmitteln. Tsung-Miu Liu und K. K. Chen geben dann einen Überblick über den Stand der Forschung auf dem Gebiet der Arteriosklerose. Die relativ grosse Zahl von Präparaten verschiedener chemischer Konstitution und Wirkungsweise, die zur Behandlung bzw. Prophylaxe der Arteriosklerose vorgeschlagen worden sind, sich aber nicht durchsetzen konnten, zeigt eindeutig, dass dieser Forschungszweig noch in den Anfängen steckt, mit anderen Worten, dass die chemischen und biologischen Anmarschwege noch ungenügend erschlossen sind. In neuester Zeit wurden besonders Substanzen diskutiert, die sich von den Östrogenen ableiten, aber möglichst geringe östrogene Wirkung besitzen. Dem Problem der Wurmkrankheiten, die in tropischen Ländern noch ungeheure Verbreitung haben, werden zwei Kapitel gewidmet: H. A. Oelkers behandelt ihre Chemotherapie, und J. Bally legt neuere chemische Aspekte der Anthelminticaforschung dar. Sehr eingehend wird von H. Haas, H. Fink und G. Härtfelder das Placeboproblem erläutert, das bei der klinischen Erprobung von Pharmaka, speziell bei gewissen Indikationen, immer wieder eine wichtige Rolle spielt und zur Forderung einer «double blind evaluation» geführt hat. Die Autoren stellen insbesondere das von amerikanischen Forschern publizierte umfangreiche Tatsachenmaterial in übersichtlicher und anregender Weise dar. Über stereochemische Faktoren, die die biologische Aktivität beeinflussen, referiert A. H. Beckett im Kapitel *Stereochemical Factors in Biological Activity*. An zahlreichen Beispielen werden die biologischen Effekte von Wirksubstanzen in Abhängigkeit ihres räumlichen Baus erläutert. Das in dieser Beziehung besonders anschauliche und deshalb interessierende Gebiet der Steroide ist dabei jedoch etwas zu summarisch behandelt worden.

Zum Schlusse folgt eine Übersicht der neueren Arzneimittel aus den letzten fünf Jahren von W. Kunz. Die umfassende, aber zwangsläufig knappe Darstellung veranschaulicht erneut die überaus rasche Entwicklung auf dem Gebiet der Arzneimittelforschung. Diese Entwicklung geht naturgemäss überwiegend in die Breite, hat aber in einer ganzen Anzahl von Fällen auch zu völlig neuartigen chemischen Typen oder zu solchen mit bisher nicht erreichbarem Wirkungsspektrum geführt.

A. Wettstein

[1] Fortschritte der Arzneimittelforschung. Herausgegeben von E. Jucker, Vol. 1, 1959 (Birkhäuser Verlag, Basel).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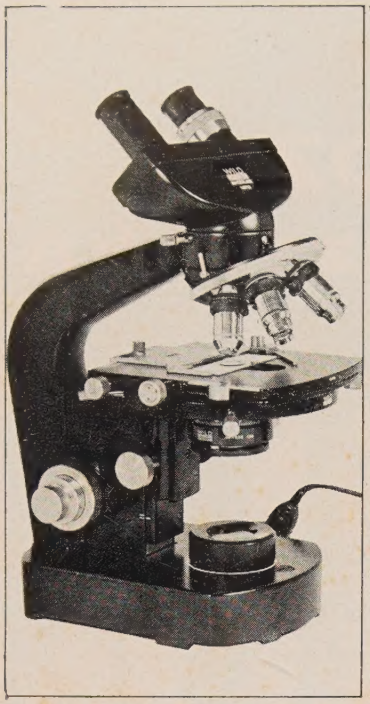

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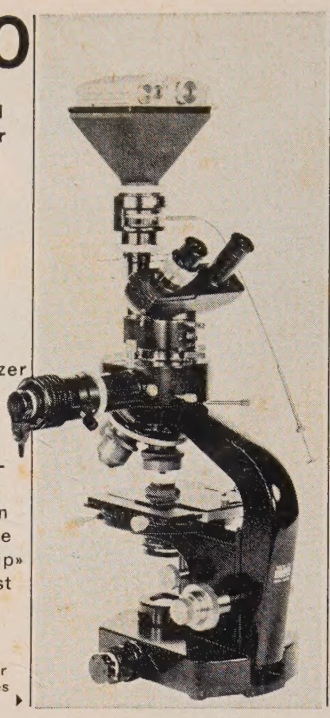

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